INFLUENCE OF DIET ON PLASMA FIBRINOGEN IN THE CHICK[1]

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The significance of physiological and pathological factors in the production of fibrinogen by Mammalia has been studied by a number of investigators (Foster and Whipple, '22 a, b, c, d; Schultz, Nicholes and Schaefer, '25; Vars, '30; Ham and Curtis, '38[2]). The studies of Whipple ('14), Meek ('12) and Foster and Whipple ('22 a, b, c, d) on varied phases of the formation of blood fibrinogen led to the important conclusion that the liver is the only potential source of fibrinogen, and more recent investigations have confirmed this view (Smith, Warner and Brinkhous, '37).

Although it was recognized at least as early as 1908 that the degree of liver injury resulting from toxic agents could be influenced by the dietary regime (Wells, '08; Davis and Whipple, '19; Miller and Whipple, '42; Neale and Winter, '38; Field, Graf and Link, '46), that dietary substances, per se, were concerned in fluctuations of blood fibrinogen was apparently first suggested by Foster and Whipple ('22 b). Their trials indicated that diets rich in animal protein, as meat,

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[2] The extensive literature on this subject is treated in the selected references mentioned here.

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liver, etc., favored higher levels of fibrinogen in the dog than diets rich in fat and carbohydrates, or fasting, alone.[3] Vars ('30) reported that high protein diets act only as a transient stimulus to fibrinogen production in the dog and he concluded that the type of protein did not play any significant rôle in determining the response. In contrast to these experimental studies, Ham and Curtis ('38) did not find that fasting or food ingestion altered the pre-test fibrinogen levels in normal human subjects. However, the ingestion by humans of moderate or large amounts of animal protein produced a mild fibrinogen response. That "active substances" in animal proteins (Foster and Whipple, '22, p. 409) are involved obtains some support in the finding of Field, Sveinbjornsson and Link ('45), that certain xanthine (purine) substances are capable of elevating the plasma fibrinogen of dogs and rabbits.

In this laboratory, an earlier investigation (Field and Dam, '45) indicated that the plasma fibrinogen of chicks could readily be altered by manipulation of the diets.[4] These results are given here, as is a résumé and additional findings, which indicate that the fibrinogen levels of avian species are influenced by dietary means in a manner similar to Mammalia.

METHODS

Approximately 200 day-old White Leghorn chicks were used in these investigations. Five to ten chicks were usually placed in each experimental and control group. They were fed either of two diets, a commercial growing chick ration or synthetic ration (table 1).

Blood samples were taken at weekly intervals. Nine-tenths milliliter of blood was withdrawn by cardiac puncture without anesthesia into a syringe containing 0.1 ml of 0.1 M sodium oxalate. Control fibrinogen, plasma protein, hematocrit de-

[3] An historical review is given in Davis and Whipple ('19).

[4] In this communication we prefer to express our findings in terms of plasma fibrinogen, the precursor of the gel-protein, fibrin. It has been customary to consider fibrin and fibrinogen as similar chemical entities (protein), the significant difference being simply a physical insolubility of the fibrin.

terminations and weight measurements used as criteria indicated that the sampling procedure provoked no abnormalities.

Total plasma protein was determined by a standard colorimetric procedure (Greenberg, '29). Plasma fibrinogen was determined by a modification (Reiner, '41) of the colorimetric method of Folin and Ciocalteu ('27) as measured with a photoelectric colorimeter. However the fibrin clot obtained with chick plasma could not be manipulated with a stirring rod as is done in handling mammalian plasmas. The clot,

TABLE 1

Composition of basal rations.

STOCK RATION [1]	SYNTHETIC RATION	gm.
Corn meal	Casein (alcohol-extracted)	15
Hominy feed	Dried yeast (ether-extracted)[2]	10
Pulverized oats	Salt mixture [3]	7.2
Pulverized barley	Gelatin	8
Wheat bran and middlings	Gum arabic	5
Soy bean oil meal	Choline chloride	0.1
Alfalfa meal	l-cystine	0.1
Meat scraps	Sucrose	54.6
Bone meal	Vitamin K substitute [4]	.001
Gluten meal	Vitamin A, D concentrate [5]	
Distiller's grain with solubles	1 drop twice weekly	
Dried skim milk and buttermilk		
Dried whey		
Limestone		
Salt plus Mn		
Fortified fish liver oil		
Wheat germ oil		

[1] Marketed as "Mystic Starting and Growing Mash" by Newman Brothers Grain Co., Rochester. The exact composition of the diet was refused. Guaranteed analysis: crude protein, not less than 20%; crude fat, not less than 3.5%; crude fiber, not more than 7%.

[2] Fleischmann type 2019.

[3] McCollum's salt mixture no. 185 7.16 gm, KI 0.966 mg, $CuSO_4 \cdot 5\ H_2O$ 9.93 mg, $MnSO_4 \cdot 4\ H_2O$, 39.8 mg.

[4] Tetrasodium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid (Synkayvite-Roche).

[5] Consists of: vitamin A concentrate from fish liver oil (containing 10^6 units per gm) 0.300 gm. vitamin D concentrate (Delsterol, containing 200,000 chick units per gm) 0.200 gm, oleic acid 24,500 gm. The concentrates were obtained from Distillation Products, Inc., Rochester. One drop (29 mg) represents 350 units A and 46 chick units D.

developed in a 15 ml centrifuge tube, was broken into small threads and fragments by vigorous stirring with the rod, centrifuged down to a mat, and both tube and clot were freed of the plasma protein in the supernatant layer by repeated washing with distilled water. The fibrin was hydrolyzed, and the color developed in a standard manner (Reiner, '41).

Fibrinogen content of plasma from chicks with avitaminosis K

The need for further modification in the procedure for measuring plasma fibrinogen of chicks became evident from a study of the fibrinogen level of chicks raised on a vitamin K-free diet. The fibrin clot obtained from these chicks was developed with difficulty and gave abnormally low values. However, this tendency was not a characteristic of the hypoprothrombinemia of a vitamin K deficiency. The plasma fibrinogen from several chicks receiving the same synthetic diet supplemented with adequate quantities of vitamin K was similarly depressed. The addition of small amounts of bovine thrombin [5] served to restore normal coagulation and give optimum fibrinogen values. Variations in the quantity of thrombin added did not measurably affect the fibrinogen values (Seegers, Nieft and Loomis, '45). The standard procedure adopted consisted of adding approximately 0.5 mg thrombin (approximately 15 Iowa units in solution with calcium chloride) to 0.4 ml oxalated plasma diluted to 10 ml with saline solution.

In several trials, duplicate determinations of the plasma fibrinogen were performed using the colorimetric procedure and a gravimetric method (Foster and Whipple, '22 a). The values obtained with both methods were in agreement.

EXPERIMENTAL

Effects of basal diets on fibrinogen levels

In the initial tests, a synthetic diet was used which differed only slightly from that given in table 1 (alcohol-extracted

[5] Parke-Davis.

casein 15 gm, ether-extracted brewer's yeast 7.5, gelatin 8, salt mixture 5, gum arabic 5, cystine 0.1, cod liver oil 5, d,l-alpha tocopherol acetate 0.005, and corn starch 54.4). This basal diet was supplemented with either 0.5 gm choline chloride, at the expense of the corn starch, or 0.003 gm of a vitamin K compound, the tetrasodium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid.[6] The fibrinogen levels of 4-week old chicks receiving the basal diet noted above, supplemented with either vitamin K or choline, and chicks receiving the natural ration, are given in table 2. High

TABLE 2

Plasma fibrinogen levels of 4-week-old chicks.[1]

DIET	AVERAGE AND S.D.[2]	RANGE
	mg %	*mg %*
Basal synthetic diet [3]	423 ± 133	285–594
Basal plus vitamin K [4]	435 ± 96	319–602
Basal plus choline [5]	505 ± 44	385–590
Control natural diet	245 ± 66	171–335

[1] Average of 9 chicks in each group.

[2] Standard deviation.

[3] Free of vitamin K and low in choline (see text).

[4] Synkayvite-Roche (tetrasodium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid) 30 mg per kg ration.

[5] Choline chloride, 5 gm per kg ration.

fibrinogen values were common to the plasma of all chicks raised on the basal synthetic diet irrespective of its choline or vitamin K content, whereas the fibrinogen in chicks raised on the natural ration was relatively low. The growth of the chicks receiving the natural ration was somewhat greater than those given the synthetic diet. After 4 weeks, the average weights were as follows: basal synthetic diet 143 gm, plus vitamin K 128 gm, plus choline 166 gm, natural ration 208 gm. The plasma prothrombin time of the chicks given either the natural ration or the synthetic diet supplemented with vitamin K was within the normal range, whereas the prothrombin time of the chicks given the unsupplemented or choline-supplemented synthetic diet was drastically prolonged (Field and Dam, '45).

[6] Synkayvite-Roche.

Interchanging rations of chicks

Two groups of chicks were fed either the basal synthetic or the natural ration (table 1) for 4 weeks. The fibrinogen levels at this time were characteristically elevated in the chicks receiving the synthetic ration, and relatively low in the chicks receiving the natural ration (table 3). When the animals were shifted from one diet to another the capacity of the synthetic ration to increase fibrinogen values appeared within 4 days while at the end of 1 week the fibrinogen levels had increased

TABLE 3

Plasma fibrinogen levels of chicks, raised on the basal synthetic and natural rations, when diets were interchanged.[1]

WEEKS ON RATION	GROUP 1 Plasma fibrinogen		GROUP 2 Plasma fibrinogen	
	Average	Range	Average	Range
	mg %	*mg %*	*mg %*	*mg %*
4[2]	437	334–509 (Synthetic ration)	187	152–202 (Natural ration)
1	301	258–360 (Natural ration)	550	397–699 (Synthetic ration)
2	332	279–395 (Natural ration)	427	359–495 (Synthetic ration)
3	263	194–365 (Natural ration)	391	345–436 (Synthetic ration)

[1] Average of 5 chicks in each group.

[2] The chicks were 4 weeks old at the time the diets were exchanged.

to 300% over the levels which the chicks maintained on the natural ration. The precipitous increase leveled off and readjustment to slightly lower values occurred in subsequent weeks (table 3). Chicks changed from the synthetic ration to the natural ration underwent a less abrupt change. The initially elevated fibrinogen values were reduced after the chicks had consumed the natural ration for 1 week, and the decrease progressed during subsequent weeks.

Effect of high vitamin K intake

The fibrinogen levels of chicks reared on diets free of vitamin K or supplemented with minimum quantities of the naphthoquinone sufficient to prevent a hypoprothrombinemia (Dam, '42) were similar (table 2). Palladin ('45) has stated that vitamin K_3 plays a rôle in the fabrication of fibrinogen, while Field and Link ('45) found that large single oral doses of various forms of vitamin K not only induce a state of temporary hyperprothrombinemia but may also temporarily elevate the fibrinogen levels in some rabbits.[7] The effect of a continued high vitamin K intake in chicks was tested by adding 0.03 gm of a vitamin K-substance [8] to a kilo of the natural (vitamin K-containing) ration given 3-week old chicks. The fibrinogen levels of the chicks were increased after they had consumed the supplemented ration for 14 days (starting plasma level 289 mg %, after 14 days 365 mg %), but thereafter fell to the pre-test normal values. Increasing the added vitamin K to 0.06 gm per kilo of ration had no further influence on the plasma fibrinogen levels.

Effect of different animal proteins

By simultaneously replacing 15% of the casein and 15% of the sucrose in the synthetic ration (table 1), the following animal protein substances were tested at a dietary level of 30%: whole dried hog liver,[8] and whole dried beef liver.[8] In addition, one group was given a diet containing 30% casein, and another group of chicks was placed on a diet in which the protein consisted of 15% casein plus 15% whole dried beef liver. The effect of these diets on the growth, total plasma protein and plasma protein is presented in table 4. The chicks given the ration containing the hog liver did not survive more than 4 weeks, prior to which the plasma protein and fibrinogen were markedly decreased. Although chicks given the diets containing either beef liver or casein (30%) exhibited the

[7] Field, J. B., and K. P. Link, unpublished experiments.
[8] Armour.

same plasma protein values, they differed in their plasma fibrinogen content. The fibrinogen levels of chicks given the 30% casein diet remained relatively low while the plasma fibrinogen of chicks given the beef liver diet increased to levels approximating those obtained when the 15% casein diet was fed (tables 2 and 3). The protein content of the regular synthetic diet (15% casein) was also increased by replacing 15% sucrose with beef liver. However, this diet did not prevent some increase in plasma fibrinogen above the level attained with the 30% casein diet (table 4).

TABLE 4

Effect of synthetic diets containing different proteins on the average growth, total plasma protein and plasma fibrinogen of chicks.[1]

AGE WEEKS	30% HOG LIVER			30% BEEF LIVER			30% CASEIN			15% CASEIN — 15% BEEF LIVER		
	Weight (*gm*)	Total plasma protein (*gm %*)	Fibrinogen ± S.D.[2] (*mg %*)	Weight (*gm*)	Total plasma protein (*gm %*)	Fibrinogen ± S.D. (*mg %*)	Weight (*gm*)	Total plasma protein (*gm %*)	Fibrinogen ± S.D. (*mg %*)	Weight (*gm*)	Total plasma protein (*gm %*)	Fibrinogen ± S.D. (*mg %*)
1	59.3			64.2			60.9			65.4		
2	72.8		215 ± 29	78.7		263 ± 54	88.9		170 ± 38	96.7		218 ± 26
3	101.9	2.9	180 ± 38	135.2	3.3	362 ± 117	120.6	3.5	175 ± 48	155.6	3.9	275 ± 41
4	128.0	1.6	169 ± 27	195.4	3.1	308 ± 31	171.9	3.3	224 ± 32	214.7	3.4	344 ± 21
5	Dead			238.7	3.4	342 ± 20	211.3	3.5	217 ± 97	280.7	3.6	338 ± 37
6				286.0	3.4	354 ± 39	246.3	3.6	219 ± 44	337.1	3.7	295 ± 43

[1] Ten chicks in each group.
[2] Standard deviation.

Effect of low protein and low casein diets

A group of ten chicks was given the regular synthetic ration until they attained 3 weeks of age. At this time, 5 of the chicks were transferred to the same basal ration in which the casein was withdrawn and replaced by sucrose. Thus the

only possible source of protein in this new diet was the yeast and 8% gelatin. Subsequently, the growth of the chicks consuming the casein-free ration ceased (table 5) and gross symptoms of dietary deficiency developed (loss of feathers, stunting of growth, etc.). The plasma protein of these chicks was decreased between the third and sixth weeks on this regime, but the fibrinogen level fell only slightly. During this period the plasma fibrinogen of the control chicks receiving

TABLE 5

Effect of synthetic diets, with and without casein, on the average growth, total plasma protein and fibrinogen of 3-week-old chicks.[1]

AGE WEEKS [3]	TIME ON DIET TESTED WEEKS	15% CASEIN			NO CASEIN [2]		
		Weight *gm*	Total plasma protein *gm %*	Fibrinogen *mg %*	Weight *gm*	Total plasma protein *gm %*	Fibrinogen *mg %*
3	1	153	3.3	286	133	3.0	274
4	2	193	3.3	324	126	3.0	275
5	3	242	3.3	329	121	2.6	261
7	5	383	3.2	386	116	2.8	256
9	7	443	3.2	370	127	2.9	267

[1] Five chicks in each group.

[2] The casein withdrawn from the diet was replaced by sucrose. A small amount of protein in the remaining formula was provided by gelatin and the yeast.

[3] All chicks were raised from hatching until 3 weeks of age on the regular synthetic ration.

the 15% casein ration was somewhat increased (table 5). When a similar ration containing only 5% casein was given to another group of 1-day old chicks, the fibrinogen levels were, by comparison with chicks of the same age receiving other diets, strikingly elevated within 1 week (table 6). Blood samples withdrawn at weekly intervals thereafter revealed a reduction in total plasma proteins while the average plasma fibrinogen levels were elevated.

Effect of fasting

The stimulating effects of diet on the fibrinogen levels in dogs can be removed by the withdrawal of food for a period

of approximately 5 days (Foster and Whipple, '22 b; Vars, '30). This procedure reduced the plasma fibrinogen to a stable low uniform level. One group of 8-week-old chicks raised on the synthetic diet was fasted for 5 days during which it had access only to water. As a result of fasting the following changes occurred: the body weight was reduced to 332 gm from 443 gm; total plasma proteins were reduced to 2.6 gm % from 3.5 gm %; and plasma fibrinogen was reduced to 222 mg % from 286 mg %.

TABLE 6

Effect of different diets on the average growth, total plasma protein and fibrinogen of chicks.[1]

AGE WEEKS	NATURAL RATION			SUPPLEMENTED NATURAL RATION [2]			SYNTHETIC DIET PLUS VITAMIN E [3]			SYNTHETIC DIET MINUS VITAMIN E			SYNTHETIC DIET LOW CASEIN [4]		
	Weight (gm)	Total plasma protein (gm %)	Fibrinogen (mg %)	Weight (gm)	Total plasma protein (gm %)	Fibrinogen (mg %)	Weight (gm)	Total plasma protein (gm %)	Fibrinogen (mg %)	Weight (gm)	Total plasma protein (gm %)	Fibrinogen (mg %)	Weight (gm)	Total plasma protein (gm %)	Fibrinogen (mg %)
1	65	2.9	222	65	3.5	215	55	3.0	221	54	—	—	41	3.2	317
2	113	3.1	228	118	3.4	211	85	3.2	283	84	3.3	228	44	2.8	307
3	185	3.0	222	192	3.1	233	117	3.3	405	110	3.1	349	51	2.4	277
4	260	3.0	222	252	3.1	265	142	3.3	455	124	3.4	414	55	2.8	429

[1] Five chicks in each group.

[2] Natural ration plus 3% liver fraction L and 5% yeast.

[3] 10 mg d,l-alpha tocopherol acetate (Ephynal-Roche) per 100 gm diet.

[4] Containing only 5% casein, sucrose replacing the 10% casein withdrawn from the regular synthetic diet.

Effect of miscellaneous rations

In table 6 are presented data obtained from chicks given varied diets; the natural ration, the natural ration to which was added both 5% yeast and 3% liver fraction L,[9] and the synthetic ration with and without vitamin E. The supplemented natural ration gave results very similar to the natural

[9] Wilson Laboratories.

ration alone. The protein levels obtained in chicks receiving the synthetic ration with or without vitamin E were essentially the same. In further trials, liver fraction L at a level of 3% was incorporated into the synthetic ration consumed by 8-week-old chicks, and brewer's yeast, at a 5% level was added to the natural ration consumed by a like group of chicks. The fibrinogen level of the chicks consuming the supplemented synthetic ration remained elevated, that of the chicks receiving the natural ration was not increased by the added yeast.

DISCUSSION

The facility with which levels of fibrinogen, a plasma globulin, can be influenced by the dietary regime is greater than has been generally recognized. By contrast, the serum globulins are relatively inert to the effects of the diet. It may be of significance that the latter probably originates from several organs or tissues while fibrinogen production is most likely an exclusive function of the liver (Madden and Whipple, '40). Although the level of plasma prothrombin in some species readily responds to diverse stimuli and influences (Link, '43–'44) the prothrombin levels of the chicks studied in similar trials (Field and Dam, '45) do not indicate that detectable differences in prothrombin levels can be induced in normal chicks (supplied with adequate amounts of vitamin K) even by drastic alterations in the dietary intake.

In a recent report, Zeldis et al. ('45) record observations of a three-fold increase in the plasma fibrinogen of a dog maintained on a low protein diet for a prolonged period. Similarly, the present studies with chicks indicate that the level of plasma fibrinogen directly reflect the nature and relative quantity of the dietary protein. A basal synthetic ration with 15% casein such as that employed in this study has found extensive use in the past and yet the chicks raised on this ration evidence high fibrinogen levels. Casein-free (low-protein) or low-casein low-protein diets resulted in some elevation of fibrinogen levels, while lower fibrinogen levels are characteristic of chicks consuming the same ration containing

30% of casein, or a natural ration containing approximately 20% mixed proteins. Thus, it is proposed that high fibrinogen levels in chicks may be a manifestation of an inadequate protein intake. Since fasting alone reduced the plasma fibrinogen to levels attained on the high casein and natural rations it would appear that a stimulus to fibrinogen production stems from consumption of diets in which the protein is inadequate, qualitatively or quantitatively. Prolonged consumption of protein-poor diets has been shown to produce liver cirrhosis in some species, and one ready manifestation of liver injury is an elevation of the plasma fibrinogen levels (Foster and Whipple, '22 b). In the rapidly growing chick, a light degree of protein inadequacy might be rapidly reflected in a hepatic fibrinogenic response. Older chicks can be made to show an elevation in fibrinogen levels less readily than young chicks when given the 15% casein diet. The high requirement of the growing chick for certain amino acids such as methionine, glycine, arginine (Hegsted et al., '41) suggests that the effect of supplementing this diet with selected amino acids, or combinations of these, on the elevated fibrinogen levels bears further investigation. Full rationalization of the response and its importance in the economy of the animal is not yet at hand.

The present data do not support the view that animal proteins, per se, stimulate the production of plasma fibrinogen (Foster and Whipple, '22 b; Vars, '30). A high and adequate casein consumption did not stimulate fibrinogen production in the chick. Thus it is more plausible that certain protein-rich materials contain substances (purines (?) Field, Sveinbjornsson and Link, '45), which are capable of inducing this effect. Whole dried hog liver in contrast to whole dried beef liver (incorporated into a synthetic diet at a level of 30%), resulted in relatively low plasma fibrinogen levels (concurrent with falling total plasma protein). However, since hog liver is toxic to vitamin E-deficient chicks (Dam, '44), and was incapable of supporting chicks in the present trials, it appears likely that lower fibrinogen levels obtained on diets containing this tissue may be entirely attributable to a toxic action.

CONCLUSIONS

1. The plasma fibrinogen levels of growing chicks were influenced by the dietary regime. Synthetic diets containing 15% casein produced high fibrinogen levels while the consumption of a diet containing 30% of casein or a natural grain ration (20% protein) gave relatively low fibrinogen values. These changes in plasma fibrinogen were independent of the growth, and the level of total protein in the plasma of the chicks.

2. The total plasma protein of chicks consuming synthetic diets low in casein (protein deficient) or a casein-free diet, was reduced while the plasma fibrinogen was elevated. Fasting reduced elevated fibrinogen levels to the low levels characteristic of the adequate natural ration and lowered the total plasma protein.

3. When chicks consumed a natural ration to which was added relatively huge amounts of vitamin K, a temporary slight increase in plasma fibrinogen was obtained and this state persisted for 14 days.

4. While a synthetic diet containing 30% dried whole hog liver resulted in reduced total plasma protein and low fibrinogen levels, and eventually death, 30% whole dried beef liver induced elevated fibrinogen levels. A diet containing 15% casein and 15% whole dried beef liver failed to prevent the elevation in plasma fibrinogen obtained with the 15% casein diet.

5. The following substances did not influence the fibrinogen levels of chicks when they were added to either a basal synthetic diet or a natural ration: choline, brewer's yeast, liver fraction L, or alpha-tocopherol acetate.

6. It is suggested that the elevated fibrinogen levels reflect a metabolic disturbance of the liver in utilizing a dietary protein intake which is inadequate, qualitatively or quantitatively.

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OBSERVATIONS ON EFFICIENCY OF SWIMMERS AS RELATED TO SOME CHANGES IN PRE-EXERCISE NUTRIMENT[1]

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Physical performance in relation to diet has been the subject of fairly extensive investigation (for review of the literature see Keyes, '43). Muscular efficiency as related to the immediate antecedent food intake has also been studied (Carpenter and Fox, '31; Haggard and Greenberg, '35; Haldi, Bachmann, Ensor and Wynn, '38), but the effect of various foods and the amount of food eaten shortly before strenuous exercise of short duration has received only scant attention. Among athletic coaches and trainers, who might reasonably be expected to have learned from experience and observation whether athletic performance is affected by the type of meal eaten before a contest, there is a wide diversity of opinion on this subject. It has been shown recently (Haldi and Wynn, '45) that in brief exhausting exercise, work output and the onset of fatigue are not affected by the kind of food consumed 2 to 3 hours before exercise. The present experiments were undertaken for the purpose of making a similar study on the effect of variations in the caloric intake several hours before exercise.

PROCEDURE

Swimming a hundred yard sprint is well adapted for studying any changes that might occur in the capacity for work dur-

[1] The expense of this investigation was defrayed by a grant-in-aid from the Sugar Research Foundation.

ing strenuous exercise of short duration. The metabolic rate is raised 10 to 50 times above the basal (Karpovich and Millman, '44) and although the exercise is exhausting, it is relatively easy to provide sufficient incentive for the swimmer to make a maximum exertion throughout the exercise period. Comparable quantitative criteria of the capacity for work under different experimental conditions are afforded by the time required to swim three laps, each being one-third the total distance. The drop-off in the second and third laps as compared with the first may be taken as an objective criterion of fatigue in the course of exercise.

Twelve subjects who volunteered to take part in the experiment were selected by the swimming coach from a group of healthy young men in the Navy V-12 training unit at Emory University. All had engaged in daily compulsory swimming for some time but they were nevertheless subjected to further intensive training for the experiment. Although there was practically no improvement in their performance from day to day, they were none-the-less eager to improve and readily responded to the urging that was given them before each experiment to try for a new record. The V-12 training program provided a highly desirable uniformity of living conditions. There were some variations in the usual daily activities over the week-end and on this account the experiments were conducted only on Tuesdays and Thursdays.

On the days of the experiments either a specially prepared light or heavy meal was served in the mess hall at 4:30 P.M. The composition of the meals is presented in table 1. To study the effect of additional nutriment in the form of readily absorbable carbohydrate the light meal was supplemented on certain days by 50 gm of cane sugar 1 hour before swimming and on other days by 100 gm of sugar. The order of the first experiments was as follows: 1, heavy meal; 2, light meal; 3, light meal plus 50 gm of sugar; 4, light meal plus 100 gm of sugar. These four different experiments will be referred to as a set of experiments. In each set the order of the preceding set was reversed to offset any error that might be intro-

duced if there should be a gradual improvement in the performance as a result of the repetitious efforts of the swimmers to make a new record.

On the day of the experiment the subject reported at the pool at 7:00 P.M. This hour of the day was chosen so as not to interfere with the trainee's classwork and other assign-

TABLE 1

Composition of "heavy" and "light" meals.

FOOD	SERVING	CHO	PROTEIN	FAT	CAL.	SERVING	CHO	PROTEIN	FAT	CAL.
		gm	gm	gm			gm	gm	gm	
		HEAVY MEAL					LIGHT MEAL			
Steak	10 oz.	0	82	26	562	6 oz.	0	49	14	322
Bread	1 slice	13	2	0	60	1 slice	13	2	0	60
Butter	14 gm	0	0	12	108	7 gm	0	0	6	54
Egg (hard boiled)	1	0	7	5	73	½	0	4	3	43
Green peas	4 oz.	11	4	0	60	2 oz.	6	2	0	32
Tomato	1 slice	1	0	0	4	1 slice	1	0	0	4
Mayonnaise (on tomato)	2 soup spoons (level)	0	1	16	148					
Peaches (canned)	1 half	8	1	0	36	1 half	8	1	0	36
Total		33	97	59	1051		28	58	23	551
Total cal.		132	388	531	1051		112	232	207	551
% of total caloric intake		13	37	50	100		20	42	38	100

ments. A blood sample was obtained by a finger puncture and the subject warmed up by swimming slowly 100 yards. The warming up exercise was followed by a brief rest period at the conclusion of which the subject toed the mark as in the usual swimming meet and at a given signal dove into the

pool. While swimming he was continuously exhorted to swim faster. Each of the laps of 33⅓ yards was timed in split seconds. At the conclusion of the swim the subject was given 3 to 5 minutes to regain his wind and then another finger puncture was made. The blood samples drawn before and after the swim were analyzed for sugar by the Hagedorn-Jensen procedure (Peters and Van Slyke, '32). The purpose of obtaining the blood samples was to determine whether there was any correlation between the blood sugar level and the food intake before exercise. In the event of such a correlation it would be of further interest to ascertain whether there was any relationship between the blood sugar level and swimming performance.

RESULTS

The swimming time and the drop-off in the second and third lap as compared with the first was the same regardless of the antecedent nutriment. The experimental data are presented in table 2. The blood sugar concentration was also the same

TABLE 2

Swimming time and blood sugar concentration 2½ to 3 hours after a heavy meal, a light meal, and a light meal when sucrose was ingested 1 hour before swimming.[1]

NUTRIMENT BEFORE SWIMMING	TIME				DROP-OFF		BLOOD SUGAR	
	1st 33⅓ yards	2nd 33⅓ yards	3rd 33⅓ yards	100 yards	2–1 33⅓ yards	3–1 33⅓ yards	Before swim	After swim
	seconds	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*mg %*	*mg %*
Heavy meal	20.5	25.8	29.1	75.4	5.4	8.6	126	148
Light meal	20.6	25.8	29.5	75.9	5.3	8.9	121	142
Light meal + 50 gm sucrose	20.6	25.9	29.3	75.8	5.3	8.7	128	139
Light meal + 100 gm sucrose	20.7	25.7	29.3	75.7	5.1	8.6	124	141

[1] Each value in the table is an average of 53 experiments except the blood sugar concentrations which are averages of 45 blood samples.

before swimming in the different experiments. There was invariably a rise in the blood sugar after swimming, but the extent of the rise was the same on the various food intakes.

All the averages in the table on the swimming time are derived from 53 experiments. It was our original intention to do 5 experiments on 12 subjects after each type of feeding which would have given 60 determinations on each desired datum. On a few occasions, however, a subject was unable to swim in the scheduled experiment. Absence from one experiment automatically disqualified him from taking part in the other 3 experiments of the set. The averages in the tables are therefore strictly comparable as they were all obtained from the same number of experiments and on the same subjects. Eight blood samples were lost, and in accordance with the rules of our procedure the samples on the other 3 experiments in the set were discarded. Accordingly each of the averages on blood sugar concentration was obtained from 45 experiments.

DISCUSSION

It is a reasonable assumption that the energy reserves of a well developed and properly nourished individual are adequate for strenuous exercise of short duration. There are some, however, who believe on a purely empirical basis that even in short bouts of exercise one's capacity for work is related to the amount of food eaten before exercise. One short distance swimmer who had been a successful contestant in the Olympics, for example, informed one of us (J.H.) that he always ingested some dextrose an hour or so before an event. His "experience" had convinced him that the extra energy supplied by the sugar enabled him to swim faster and with less fatigue. Similar opinions have been expressed by various athletic coaches. There are those on the other hand who have the definite impression that they perform best in athletic exercise after a light meal (Hellebrandt and Karpovich, '41). This point of view is probably based on the assumption that after a heavy meal a distended stomach may interfere with

the descent of the diaphragm or that digestive and other processes might interfere with the physiological adjustments that take place in exercise. These conflicting empirical deductions suggested the advisability of obtaining precise information by experimental studies.

The present experiments show that performance in severe exercise of brief duration is not affected by the amount of nutriment taken 2 to 3 hours before exercise. In previous experiments it has been found that swimming performance is not affected by the kind of food eaten beforehand (Haldi and Wynn, '45). It need not be emphasized that without further experimentation these conclusions can not be regarded as applicable to long protracted and exhausting exercise.

The decrease in speed which may be quantitated as the drop-off in the second and third laps as compared with the first serves as an objective criterion of fatigue. Obviously, fatigue during swimming, as measured by this standard is not related to the amount of food taken before swimming nor to a fall in the blood sugar while swimming, since the blood sugar concentration was higher immediately after than before exercise. It is worthy of note that the blood sugar level before swimming was the same when 50 or 100 gm of sucrose had been ingested approximately 1 hour after the meal as when no sugar was taken. This may have been due to the same mechanism which prevents a rise in blood sugar when the second ingestion of glucose follows the first within an hour and a half (Foster, '23).

A rise in the blood sugar concentration after severe exercise has been observed also by other investigators. From observations on football players, the conclusion has been offered that hyperglycemia is uncommon in exercise with little or no emotional stress but common in exercise with emotional stress on the football field (Edwards, Richards and Dill, '31). This point of view, however, does not appear to have been substantiated by later experiments (Dill, Edwards and Mead, '35). Blood sugar concentration was found to be increased 10 to 66% when the subjects performed work that brought on

exhaustion within 10 to 40 minutes. The type of work done was of a nature that should not have been conducive to emotional stress. It appears likely that the high blood sugar level immediately after swimming in our experiments may have been a direct effect of exercise. The subjects did not experience the emotional states that are usually aroused by competitive games and a cheering crowd. As far as could be ascertained the determined effort of the swimmer to excel his previous record did not cause any marked emotional excitement.

SUMMARY AND CONCLUSIONS

A study of the performance of swimmers in a hundred yard sprint has been made to determine the influence of the size of the antecedent meal on severe exercise of short duration.

Two and a half to 3 hours after a heavy meal, the time required to swim each of three laps in the hundred yard sprint was the same as when a light meal was taken.

Supplementation of the light meal by the ingestion of 50 or 100 gm of sucrose before swimming had no effect on the swimming time.

The drop-off in the second and third laps which is taken as an objective index of fatigue was the same regardless of the amount of the food intake before swimming.

The blood sugar level before swimming was the same 2 to 3 hours after the various amounts of antecedent food intake. Immediately after swimming there was a rise in the blood sugar which was practically the same in all experiments. This rise in blood sugar was apparently a direct effect of exercise and not due to emotional stress.

It is concluded that there is no relationship of speed, power and skill in swimming a hundred yard sprint to the amount of nutriment taken 2 to 3 hours before swimming.

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BIOLOGICAL VALUE OF PROTEINS IN RELATION TO THE ESSENTIAL AMINO ACIDS WHICH THEY CONTAIN[1]

I. THE ENDOGENOUS NITROGEN OF MAN

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TWO FIGURES

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The biological value method introduced by Thomas ('09) has undergone many improvements and modifications in its various applications. As Mitchell has made clear in his many articles describing improvements for application to rats and farm animals and summarized as to the fundamental principles involved in several searching reviews ('26, '42 and '44), it is not, strictly speaking a method for measurement of the biological value of a protein for maintenance except when applied to, and based on, the endogenous nitrogen excretions of adult animals.

In consideration of physiological differences between man and lower animals with respect to his tolerance of foods, this laboratory developed the use of egg protein metabolism as a relative basis, in contrast with the endogenous or absolute basis (Sumner, Pierce and Murlin, '38; Murlin, Nasset and

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Rochester.

Marsh, '38; Murlin, Marshall and Kochakian, '41). This modification was perfectly justified also by the high absolute biological value of this protein.

In undertaking the present studies on human subjects, we visualized an opportunity for so ordering the sequence of periods of observation as to permit the development of conversion factors which would relate values referred to endogenous excretion with those referred to egg, or any other high value food protein as base. The second paper will be concerned with this question of the interconvertibility of biological values.

The employment of the endogenous base presented other advantages aside from that of convertibility; viz., economy in the use of the very expensive synthetic amino acids; and the comparison of our results with those obtained by the endogenous method, principally on rats, in other laboratories.

A long series of studies on the same subjects was hoped for, involving perhaps the necessity of many no-protein periods; and it was feared these hopes could not be realized without encountering the risk of serious depletion of reserve protein, even to the point of liver damage, unless a reliable measure of endogenous nitrogen excretion could be accomplished in relatively short periods. It was necessary, therefore, to give consideration to the factors affecting the attainment of reliable endogenous values in the adult man.

BASAL DIET AND SEQUENCE OF PROTEINS

Table 1 exhibits the items of a diet in which egg protein furnished 95% of the nitrogen after deducting the nitrogen of coffee, tea and the caffeine in a bottle of carbonated beverage. As a rule the total energy value of the diet at the beginning of a series was placed at 45 cal. per kilo of net body weight, but when in individual cases it became apparent that this allowance was not sustaining body weight it was raised to 48 cal. per kilo or sometimes higher, by addition principally of carbohydrate. Even with all the carbohydrate the subjects could tolerate, however, some loss of weight could not always

be prevented. It was always heaviest in periods when amino acids were being fed.

Arrowroot starch was definitely superior to either corn starch or wheat starch as the principal ingredient of the biscuit or muffin because of its lower nitrogen content and better acceptability.

TABLE 1

Illustrative diet, used by F. L., weighing 66 kg. At the beginning this diet contained 4.75 gm N, 378 gm carbohydrate, 149 gm fat, and furnished 2970 Cal.; later, when the Cal. were raised to 3168 (48/kg), it contained 404 gm carbohydrate and 159 gm fat.

COMPONENT	AMOUNT	N	FAT	CARBOHYDRATE	VITAMINS PER DAY	
	gm	gm	gm	gm		
Egg	228	4.55	24		A (Navitol)	4,000 I.U.
Butter	40	0.009	40		D (Navitol)	500 I.U.
French dressing	30	0.006	23		Ascorbic acid	100 mg
Lettuce	80	0.077		3	Thiamine HCl	1.0 mg
Sugar	15			15	Riboflavin	0.8 mg
Marmalade					Niacin	10.0 mg
Biscuit (arrow-root starch)	(4 pieces)	0.084	55	148	Pyridoxine	0.5 mg
					Ca pantothenate	2.0 mg
Orangeade (artificial)	408	0.0088		102	Paramino benzoic acid	0.8 mg
Candy	80			80		
Coffee (2 cups)	360	0.1836			Choline HCl	1.5 gm
Tea (1 cup)	180	0.144			Inositol	100.0 mg
Cola drink	180	0.006		18		
Omelet oil	17		17			
Applesauce	100	0.0193		38		
Vitamins		0.0126				
Mineral mixture [1]		0.0002				
Total		5.101	159	404		
Deducting for N in coffee, tea and cola		— 0.334				
		4.767				

[1] The mineral mixture had the following composition in gm (for 300 man-days): $CaCO_3$, 600; $MgCO_3$, 78; $MgSO_4$, 30; NaCl, 900; KCl, 345; KH_2PO_4, 627; $FePO_4 \cdot 4H_2O$, 20; KI, 0.08; $MnSO_4$, 0.35; NaF, 1.00; $Al_2(SO_4)_3 \cdot K_2SO_4$, 0.17; $CuSO_4$, 0.90. (Partly from Mattill and Mattill, Endocrinology and Metabolism, '23, and partly from Hubbell, Mendel and Wakeman, J. Nutrition, vol. 14, p. 273, '37.)

The typical sequence of periods which prevailed in more than half the series listed in table 2 started with egg protein, the distribution of calories being set at 4% for protein, 51% for carbohydrate and 45% for fat. When endogenous nitrogen was to be determined the "no-protein," consisting of the same diet except egg, followed immediately after the egg period, adjustment of calories for loss of the protein being made. Then came the test protein fed at some definite level with reference to the endogenous N, and following this came the amino acid mixture compounded in a definite relationship to the composition of the test protein. A new cycle beginning with egg protein then was started.

The subjects of these experiments were principally conscientious objectors assigned to the project by Selective Service from the quota allocated to O.S.R.D. and finally numbering 21 men and one parolee. Sixteen other men and 10 women, volunteers from the Medical School and Strong Memorial Hospital personnel, served exclusively in series 1 and 2, and in small numbers in series 3, 4 and 7.

All but one of the subjects were in apparently good health when entering upon the experiments and, not counting minor ailments, all but three continued to the end in the series to which assigned without detriment to health. The three exceptions are mentioned in connection with the discussion in this paper of the series to which they belonged.

Continuous determinations of nitrogen by the macro-Kjeldahl method were made in all principal items of the diet and in all new lots of even minor items as well as in every day's 24-hour urine and period feces. Creatinine N was regularly determined as a check on collection of urines, and amino acid N was run in the urines of several periods in which they were fed.

FACTORS AFFECTING THE ENDOGENOUS LEVEL OF EXCRETION

(1) The starting level

Folin ('05), in changing four subjects from a general mixed diet containing from 14 to 16.8 gm of N to his starch-cream

diet containing less than 1 gm of N, found that the lowest level of uric acid N was reached on the fourth, fifth, first and third day, respectively, for his four subjects; the lowest urea nitrogen was reached on the seventh, fourth, fifth and fifth day, respectively, and the lowest total N on the tenth, seventh, fifth and fifth, respectively. On the first and second of the four subjects the observations were terminated on the tenth and seventh days; consequently it is uncertain whether or not the total nitrogen had reached a steady state. But for the other two cases it certainly did not descend farther on the sixth and seventh days after starting the low N diet. Martin and Robison ('22) found that their own N-excretion fell in 5 days from 8.61 gm to 2.48, and from 8.79 gm to 1.99 daily for the two authors, respectively, when they started a "no-protein" diet.

In the present series of studies the "no-protein" period always changed the nitrogen intake abruptly from a level varying from 3.8 to 5.5% (of calories) for protein in the diet (see table 2), or from 4.5 to 6.7 gm N daily. The "no-protein" diet never contained more than 0.43 gm N, average for the squad, and was as low as 0.195 gm daily. Hence the absolute change was of the order of 4.3 to 6.3 gm N — a mean drop but little more than a third of that imposed by Folin. Expressed in percentage the average, however, was 94 compared with 93 in Folin's subjects. The starting level having been maintained usually for several previous 5-day periods and having often approximated much more nearly the endogenous level of excretion, it is fair to assume that much less reserve protein was present in these subjects than in Folin's and therefore that they would reach the endogenous level of excretion considerably earlier. This proved to be the case. In an early group of eight subjects, the average difference of urinary N between the second and third "no-protein" days was 0.378 gm and for half the squad who continued to the fourth day the difference between the third and fourth days was only 0.157 gm. In the next squad the third-day excretion proved to be slightly higher on the average than the second.

It was therefore decided to accept tentatively the excretion of the third day on "no-protein" as approximating closely enough the true endogenous level to insure dependable comparisons of the biological values of test proteins and corresponding mixtures of amino acids fed a little above this level. Later it was found, as expected, that the position of the no-protein period in a series of periods on comparatively low protein determined the reliability of the endogenous level of excretion attained by the third day. These facts will be illustrated below.

(2) Position of the no-protein period in the series and nature of the protein preceding it

A summary of the average endogenous nitrogens on the third day for 12 no-protein periods, and on the fourth day as well for four such periods is presented in table 2. The average daily fecal nitrogen excretions for the entire periods are included under the heading "endogenous" with full realization that the fecal nitrogen cannot be all "metabolic," to use Thomas' awkward term, but derives principally from residues of the digestive secretions and from exfoliated epithelial cells much of it transmuted into bacteria.[2]

In column 2 of table 2 is given in Roman numerals the number in each series, of the "no-protein" periods. It can readily be seen that later such periods in series 5, 6 and 8 (the only ones in which more than one occurs) gave the lower urinary nitrogens, and in 2 out of 3 of the series lower fecal nitrogens as well. This lower excretion rate, however, might well be influenced by the nature of the food protein which preceded the "no-protein" period in any given case, the level at which it was fed, and the length of time. Columns 3, 4 and

[2] For this reason this laboratory has preferred the term "alimentary nitrogen" which at least correctly indicates its origin if not its exact nature. It is a loss from the body (alimentary organs) incurred in the processes of digestion of the indispensable energy-giving foods and must be made good for maintenance. In that sense it represents maintenance as truly as do the products of catabolism of hematopoetic organs or the production of blood corpuscles themselves, the overturn of which must represent no small part of endogenous urinary nitrogen.

5 supply information with respect to these factors. Fortunately in series 6 and 8 the first two of them could be made identical; namely, whole egg protein and 5%-of-calorie level in all three periods. This circumstance leaves the factor of

TABLE 2

Endogenous nitrogen excretion following different proteins and at different times in a series.

SERIES NO.	NO-PROTEIN PERIOD	PROTEIN PRECEDING NO-PROTEIN	PROTEIN LEVEL AS % OF CAL.	NO. OF DAYS OF THIS PROTEIN	AVERAGE ENDOGENOUS NITROGEN PER DAY		SUBJECTS		COEFFICIENT OF ENDOGENOUS N IN MG/KG OF BODY WEIGHT	
					Urine	Feces	No.	Body wt. (av.)	Urine	Feces
					gm	*gm*		*kg*		
2	VI	Whole egg	4.0	4	3)[1]2.678	0.888	6	60.8	41.7	14.5
3	X	Amino acid mixture	3.8	5	3) 2.350	0.822	7	63.8	37.7	12.9
4	VI	Whole egg	4.0	6	3) 2.629	1.074	11	59.8	42.9	18.0
5	II	Whole egg	4.0	8	3) 2.642	1.201	10	66.6	39.6	18.0
	VII	Beefsteak	5.5	3	3) 2.403	0.959	10	66.0	36.8	14.5
6	II	Whole egg	5.0	11	3) 2.203	1.082	4	62.9	35.0	16.0
	VI	Whole egg	5.0	6	3) 2.131	1.227	4	63.0	33.8	19.5
	X	Whole egg	5.0	6	3) 2.169	1.288	3	64.6	33.6	20.2
8	II	Whole egg	5.0	9	3) 3.177	1.270	8	66.2	48.0	19.2
					4) 2.947			66.2	44.6	
	VI	Whole egg	5.0	7	3) 2.596	1.064	8	65.2	39.8	16.5
					4) 2.149			65.0	33.1	
	XII	Whole egg	5.0	7	3) 2.063	1.028	8	64.3	32.1	16.3
					4) 2.042			64.1	31.7	
9	VIII	Wheat germ	6.0	4	3) 2.379	1.026	6	68.1	34.9	15.1
					4) 2.050			68.1	30.1	
		Weighted av. 85 cases			3) 2.502			3)64.3	38.9	
		Weighted av. 22 cases			4) 2 083			4)65.5	31.8	

[1] 3) and 4) refer to days of no-protein period.

position unencumbered except for the length of time the preceding protein was fed. The latter may be resolved at once by pointing out that "no-protein" II necessarily is the one following the introductory protein after these squads had been for at least 2 months (series 6) on ad libitum diets. It was necessary therefore to prolong period I until the excretion

level no longer reflected the higher protein ingestion. A shorter period would certainly have produced a still higher "endogenous" excretion on any early day.

The issue with respect to the nature of the preceding protein (meaning its biological value) demands closer attention in the case of series 5, where preceding period II whole egg was fed for 8 days at a 4% level and preceding period VII beefsteak at 5.5% was fed for only 3 days. It appears that higher level could not compensate for lower value and a shorter period in sustaining the N excretion. In accordance with the usual interpretation this means that the protein reserves of the body had not been so well replenished by beefsteak as by egg, consequently were more quickly exhausted. Possibly a period of feeding equal to that of the egg period would have given a different result, to which the answer, not wholly adequate, is that beefsteak was itself preceded by egg for 2 days. Here we get into the question of more antecedent conditions than the diet immediately preceding, and for further light on this situation we must invoke a factor which is cumulative (table 3). The possible influence of an amino acid mixture (series 3), and of wheat germ, supplying a comparatively low value protein (series 9) likewise cannot be resolved without reference to antecedent conditions because each of these stands alone in the series; there is no endogenous period with egg as "supporting protein" preceding it with which either of these foods can be compared for the same squad of subjects.

Meantime it is noteworthy how remarkably well the "endogenous" urinary nitrogens agree following egg at 4% level, in two periods VI (series 2 and 4) and one period II (series 5). The average third-dav excretions from two entirely different squads and a repetition with one, launched into no-protein from the same platform, so to speak, were 2.678, 2.629 and 2.642 gm N, respectively. How is it possible that egg protein could exert so constant an influence? The level of feeding was the same in the 3 widely separated periods, but the duration of this high quality influence varied from 4 to 8 days.

TABLE 3

Relation of the sequence of proteins, and the consequent accrued nitrogen balance antecedent to no-protein, to the endogenous urinary nitrogen excretion.

SERIES NO.	PLACE OF NO-PROTEIN PERIOD	SEQUENCE OF PROTEINS FROM BEGINNING OF SERIES TO NO-PROTEIN PERIODS, AND NO. OF DAYS OF EACH	FROM BEGINNING OF SERIES TO 3RD AND 4TH DAYS OF NO-PROTEIN				AVERAGE ENDOGENOUS URINARY NITROGEN FOR	
			Total days to		Accrued N-balances on			
			3rd day	*4th day*	*3rd day*	*4th day*	*3rd day*	*4th day*
2	VI	Egg 5, soy bean 4, egg 5, soy bean 4, egg 4	24		— 11.3		2.678	
3	X	Egg 6, yeast 4, egg 5, yeast 4, egg 5, yeast 6, beef 6, egg 6, amino acids 5	49		— 25.3		2.350	
4	VI	Egg 5, yeast 4, egg 5, yeast 4, egg 6	26		— 24.1		2.629	
5	II	Egg 8	10		— 11.9		2.642	
	VII	(No-prot. 3), cotton seed 5, amino acids 3, egg 2, beef 3	26		— 17.8		2.403	
6	II	Egg 11	13		— 3.3		2.203	
	VI	(No-prot. 3), corn germ 5, wheat germ + amino acids 4, egg 6	31		— 5.9		2.131	
	X	(No-prot. 3), egg 5, amino acids 4, egg 6	49		— 10.2		2.169	
8	II	Egg 9	11	12	— 12.9	— 16.6	3.177	2.947
	VI	(No-prot. 4), corn germ 5, amino acids 4, egg 7	31	32	— 36.7	— 40.4	2.596	2.149
	XII	(No-prot. 4), wheat germ 7, corn germ 9, amino acids 4, egg 7	62	63	— 59.9	— 62.8	2.063	2.042
9	VIII	Egg 6, egg 5, veg. mixt. + soy bean 4, wheat germ 4, veg. mixt. + soy bean and wheat germ 4, egg 5, wheat germ 4	34	35	— 13.3	— 16.5	2.379	2.050

Is this factor of duration then of no consequence? The answers will be found in the next section.

(3) Cumulative antecedent conditions

From a study of the data of Mitchell and associates in four of their publications from 1926 to 1933 Ashworth ('35) found considerable evidence that the previous diet influences endogenous nitrogen of the urine in rats. If high quality protein (liver) was fed immediately before the no-protein period the endogenous N in the urine rose above the previous level; if low quality protein (flour, cocoa) preceded the no-protein period the tendency was for that N to fall below previous levels. He found evidence of the influence of dietary protein on endogenous N excretion also in the work of Mason and Palmer ('35). In his own work, however, using lactalbumin as high quality and corn gluten as low quality protein Ashworth did not find any significant difference in the effect of these two on a final endogenous period, except "when the reserve protein supply of the body was reduced to a low level by long periods on N-free diets." He concluded that long periods on low quality test proteins at low levels of intake should not be used, if a decrease in endogenous N in a subsequent period is to be avoided.

Ashworth does not suggest, nor have we seen elsewhere any proposed means of measuring the effects of previously ingested proteins by which investigators, on whom is laid a responsibility for protection of their subjects, could predict the reliability of the endogenous level attained on a given day. The accrued nitrogen balance, as a criterion suitable for this purpose, seems to meet the need. For as a rule a balance can be struck for all previous food periods before the end of the third day of the current no-protein period and one can then determine whether an additional day is required. In these studies continuous nitrogen balances were available after the first few days of each series during which the subjects were becoming adjusted gradually to low (egg) protein.

Table 3 presents (col. 3) the sequence of proteins fed, in all of the series where endogenous nitrogen was especially needed, previous to its determination. In column 4 are found the elapsed times from the beginning of the series to the third and fourth days of the no-protein period in question, and in column 5 the accrued N-balances to those points. In each sequence the last protein named is the one given in table 2, column 3.

Referring to the questions raised in the previous section, we learn from table 3 that up to the third day of no-protein period VI in series 2 and the same day of no-protein period II in series 5, the total antecedent conditions had brought the two squads to the same average nitrogen deficit; namely, 11.3 and 11.9 gm, respectively. The supporting protein and its level being the same, the same endogenous excretion resulted.

The duration of the "supporting protein" period, as we have called the one immediately preceding a no-protein period, we thus learn cannot be the determining factor because the time it was fed in one case was twice that in the other. Neither can the nitrogen debt incurred previous to no-protein be credited with sole responsibility for the urinary N output; for while this item agrees perfectly for the two periods just mentioned, we find more than double the amount (24.1 gm) had been incurred up to the third day of period VI of series 4 in which the excretion of this squad of 11 averaged 2.642 gm — very close, as just observed, to the average performance of squads 2 and 5 on the corresponding day of their first no-protein periods.

It should be pointed out that squad 5, the subjects of series 5, are the same men, less one, who made up squad 4. They concluded the latter series with an accrued deficit of 23.1 gm of nitrogen each. Then intervened a recuperation period of 10 days in which it is safe to say they ate to repletion, so that the introductory period on egg in series 5 found their reserves well stocked. A careful analysis of all the data does not reveal any hold-over effect from the deficit incurred in series 4.

Almost certainly such a condition would have caused heavy retention and a positive balance in period I. Actually this period of 8 days on egg contained two short periods in which the balances were kept and both were negative.

The best conclusion possible at the moment is that egg protein is of such a high quality in building up reserves that, regardless of the length of feeding and the antecedent conditions affecting nitrogen equilibrium, it maintains as "supporting protein" a surprisingly constant third-day endogenous excretion and, as may be seen in figure 1, one that is higher

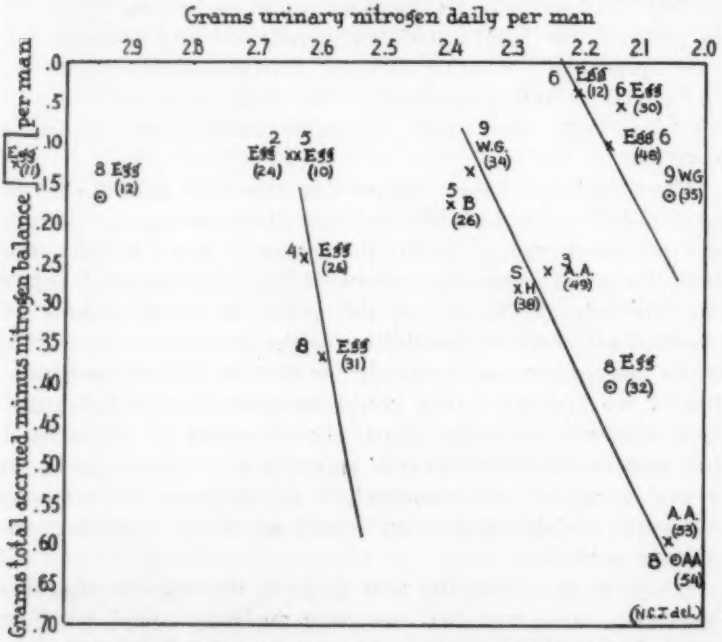

Fig. 1 Large numerals designate numbers of the squads whose daily averages are given by the coordinate points: x for third day of no-protein diet, ◉ for fourth day. A numeral in parentheses denotes number of days since beginning of the series to the day of no-protein designated. Name of protein is that given in table 2 as immediately preceding the no-protein period: W. G., wheat germ; B, beefsteak; H, halibut steak; A.A., a mixture of essential amino acids.

Halibut was fed to a special squad, designated "8", belonging to a different project.

than following any other protein we have used in that position. Four of the third-day determinations charted lie close together (to the left center) regardless of the magnitude of the accrued nitrogen deficit up to the day of measurement. The exceptional ones will be discussed in a moment.

The third-day endogenous values following other proteins used in the "supporting" position tend to group themselves to the right center of the chart and to be arranged also in a loosely linear fashion from upper left to lower right.

Series 6 deserves special consideration because it illustrates the fact that a squad of men, by subsistence over a very long period on low but fairly good protein on the average, may be brought to so low a state of reserve protein that starting as usual with egg as introductory protein and thus establishing at once a fair rate of nitrogen retention (aside from the "no-protein" periods) may still have reserves low enough to permit a steady endogenous level to be reached in 3 days. The squad started with 5 members, but one had to be excused at the end of 3 weeks because of a stomach ailment later diagnosed as gastritis. The remaining four had all been members of squads 4 and 5 (Aug. 15 to Dec. 22, 1944 with one break of 10 days) and two of them of squad 3 (June 10 to Dec. 22, 1944 with an additional break of 2 weeks). All had been off the diets from Dec. 22 to March 3.

The coefficients in milligrams of endogenous urinary N per kilo of body weight (table 2), prove that the average output for these men in period II of series 6 was already significantly lower than those of series 4, period IV, and series 5, periods II and VII, of which they had been members. Coincidentally their partial averages in the larger squads were exactly the same as those of the entire squads. It seems perfectly clear that their reserves had not been completely restored [3] when series 6 was begun.

[3] These men through January and February, 1945, had been subjects several times a week of hot-room experiments in another department, sweating profusely for many hours at a time, and had lost some weight.

Alarmed a little at this condition when it was discovered in period VI, blood serum proteins and hemoglobin values [4] were determined on them immediately after period VII with the result that all were found to be well within the normal ranges.

It is certain, therefore, that the liver and other hematopoetic tissues of these men had not suffered injury; but it is conceivable that the lower rate of endogenous excretion did not reflect a state of general depletion so much as an acquired power of conservation of such reserves as were available. The latter seems the more probable.

From March 3 until August 22 these four men continued on the low protein diets with a break of 17 days in May. Period II of series 6 found them with an accrued N-balance very near equilibrium, and their endogenous N for the third day at only a slightly higher average level than it was in period VI. In fact, as may be seen in figure 1, all three of the endogenous determinations lie very close together in the upper right-hand corner of the chart.

There is evidence in the column for fecal N in table 2 that these subjects did not digest protein so well as they had done in previous experiments; for their partial averages in this regard were also equal to those of the entire squads 4 and 5. This slightly higher excretion of fecal nitrogen, however, does not compensate for the lesser urinary excretion compared with earlier squads. It may indeed reflect only the higher intake that prevailed earlier in the supporting position.

Series 7, like series 1, contained no endogenous periods.

Series 8 started with a perfectly fresh squad of nine men, but the results are compiled for only eight of them, as one was lost by illness [5] before period XII. Since only about

[4] The authors are indebted to Mr. Robert Tully for these determinations.

[5] This man had arrived with furunculosis which persisted in spite of conservate drainage, but eventually appeared to be completely healed. Meantime, however, pain in the left ischial region developed and x-ray examination revealed "an area of bony erosions along the lateral margin of the left ischial tuberosity 1½ cm in depth," and a diagnosis of osteomyelitis was made. He was immediately admitted to the Orthopedic Division of Strong Memorial Hospital for penicillin treatment.

11 weeks remained before the then termination date of this project, we did not hesitate to subject these fresh subjects to three no-protein periods of 4 days each. The first of these found them with a slight minus balance which, augmented by the first days on no-protein, amounted to 12.9 and 16.6 gm N for the third and fourth days, respectively. Period VI began with an accrued deficit at 31 days of 36.7 and at 32 days of 40.4 gm. The endogenous urinary nitrogen on the fourth day (thirty-second from beginning of the series) approached a steady state as proved by period XII 22 days later (q.v.). The urinary nitrogens of the third and fourth days, after 53 days on comparatively low protein diets and at about 60 gm N-deficit, were equal.

Series 9 started with seven subjects, six of them CO men and one a parolee. Four were veterans from at least four earlier series, one a former subject returning after over 6 months respite, and two perfectly fresh dietary subjects. Because of the long tour of service on this project which the majority of them had given, only one no-protein period was imposed and that quite late in the series. At 32 days the accrued minus balance was only 3.79 gm and yet on the fourth day the average endogenous level of excretion was the same as for squad 8 after 51 days and an accrued minus balance of 46 gm. The difference is due to the better average state of nutrition of squad 8 at the start. The two squads were being studied simultaneously.

Summarizing this section it can be asserted that a quite definite relationship exists between the accrued N-deficit and the rate of decline in endogenous urinary nitrogen. In figure 1 this is shown by a shift to the right of all values the lower they lie in the scale of deficiency. They do not, however, fall into a single linear arrangement, but into three different slopes depending on the original state of nutrition and on the quality of the supporting protein. The fresher the subjects, following egg, the farther to the left in the chart; the more depleted at the beginning of the series, and also following egg, the farther to the right. The higher the position in the chart,

the more resistant is the reaction to length of time on low protein intakes; while the lower the position, the less resistant. The influence of biological value in the supporting position is seen with squad 5. Instead of moving downward in the chart in changing from egg as support to beefsteak, these ten men move to the right, i.e., the bottom drops out of the supporting structure, so to speak; reserves are not sufficient, or are more tenaciously held, and lower endogenous levels are attained. The effect of a fourth day as compared with the third is illustrated by squads 8 and 9. Following egg the movement from third to fourth (x to ⊙) for 8 is down the endogenous scale much more rapidly than down the deficiency scale. The same applies to 9 following wheat germ.

The chart, we believe, justifies our conservative plan with human subjects. It is not necessary to subject men at once to a long period of protein starvation, thereby possibly endangering the most valuable reserves — those which maintain blood proteins inclusive of specific protective agents. It can more safely be accomplished by a gradual approach. Keeping these men on a level of 5% of calorie intake with comparatively high value proteins for 25 to 35 days brought them to the region of relatively stable endogenous excretion with only moderate depletion, compared for example with the rate of depletion in Deuel's case (Deuel, Sandiford, Sandiford and Boothby, '28). A blood examination [4] made on squads 8 and 9 in the final periods of their respective series revealed only one man slightly below the normal range for hemoglobin and all who were examined (two from each squad did not appear) well within the normal for serum proteins.

REGRESSION OF ENDOGENOUS URINARY N ON BODY WEIGHT

Figure 2 displays the relationship of endogenous urinary N to net body weight. The statistical constants for these curves are given in table 4.

It is clear that the fourth day is less variable than the third for subjects who have been for at least 30 days on low protein diets of which at least two contained whole egg protein.

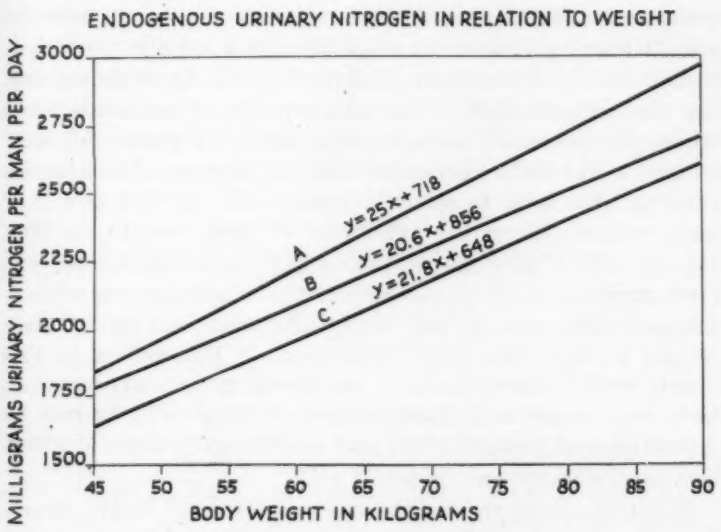

Fig. 2 Regression curves described by their respective formulae for: A, 73 determinations of third-day no-protein excretion on 28 men; B, 7 determinations of third-day no-protein excretion on 7 women; C, 22 determinations of fourth-day excretion on 14 men.

TABLE 4

Statistical data for plot of the relation of endogenous urinary nitrogen to body weight.

	THIRD DAY		FOURTH DAY
Number	73 on men	7 on women	22 on men
Standard deviation	7.05	10.30	6.70 kg
Standard deviation	315.00	332.00	222.00 mg
Correlation coef.	0.56	0.64	0.66
Regression y on x	25x + 718	20.6x + 856	21.8x + 647

RELATION TO BASAL METABOLISM

It was discovered in 1927 by Terroine and Sorg-Matter that in several species of warm-blooded animals the total endogenous nitrogen excretion bears a constant relationship to basal metabolism. This was confirmed by Smuts ('35) for mice, rats, guinea pigs, rabbits and pigs, the ratios of milli-

grams urinary (only) nitrogen to calories of basal metabolism per 24 hours giving an average for the 5 species very close to 2.0. Brody, Procter and Ashworth ('34) have shown that the basal metabolism of the whole range of mammals from mouse to elephant increases with the 0.734 power of body weight while the endogenous urinary nitrogen for a series ranging from mice to cattle increases with the 0.72 power of body weight. Since the exponents of body weight on both log-log curves give slopes practically indistinguishable one from another, the authors conclude that "endogenous urinary nitrogen increases at the same rate with increasing body weight as does the basal metabolism." Exceptions to this nearly perfect harmony in the metabolic symphony, however, have been found in different stages of immaturity in rats by Ashworth and Cowgill ('38) and at different planes of nutrition by Treichler and Mitchell ('41).

It appears from the well-known experience of Deuel, Sandiford, Sandiford and Boothby that it requires a long time for a man to reach the minimal endogenous level of nitrogen excretion. After 39 days on a protein-free diet Deuel's urinary nitrogen was still falling slowly, while in the record of squad 8 (table 3) it required a period of only 29 days' continuous subsistence on a low protein regime, introduced by a 4-day period on no-protein, to reach the stage where, on the fourth day of the second no-protein period, the excretion appeared to become stable. Certainly 1 month later the excretion on the third and fourth days gave average levels for the entire group of eight men only 0.1 gm lower.

It seems incongruous that one phase of (protein) maintenance metabolism which cannot be attained inside a month should be so definitely ("rigorously" is the term used by Terroine and Sorg-Matter) proportional to another phase (energy) of maintenance metabolism which can be attained in 15 hours.

We have not been able to spare the time or to arrange for the use of the apparatus to follow basal metabolism in all our no-protein periods, as we should have liked to do; but

through the courtesy of Mr. J. T. Anderson and his assistant Mr. Frank Hastings we are able to present the suggestive data set out in table 5. Five of the eight men in squad 8 volunteered for basal determinations before breakfast on July 10, and again on August 10, just before closing the fourth day of the no-protein periods VI and XII in series 8 (table 3).

TABLE 5

Relation of endogenous urinary N to basal metabolism.

	SUBJECT		SURFACE AREA [1]	ENDOGENOUS URINARY N IN MG/DAY	BASAL METABOLISM: CAL./DAY	ENDOGENOUS URINARY NITROGEN PER		
	Wt.	Ht.				Cal.	kg	sqm
	kg	*cm*	*sqm*					
				July 10, 1945				
B.M.	51.8	167	1.58	1748	1315	1.33	33.2	1106
W.L.	57.2	170.7	1.67	1799	1411	1.27	31.4	1077
G.S.	65.3	167.5	1.75	2187	1217	1.80	33.5	1250
L.B.	71.9	167	1.80	2188	1488	1.47	30.4	1215
M.W.	72.0	179	1.90	2327	1507	1.54	32.3	1225
Av.	63.6	171	1.74	2049	1387	1.48	32.2 ± 3.1%	1173 ± 5.6%
				August 10, 1945				
B.M.	51.8	168	1.58	1660	1294	1.28	32.0	1051
W.L.	56.4	170.7	1.66	1728	1410	1.22	30.6	1041
G.S.	63.3	167.5	1.73	1990	1306	1.52	31.4	1150
L.B.	70.0	165.5	1.77	2098	1498	1.40	29.9	1185
M.W.	70.4	180	1.89	2038	1555	1.31	29.0	1073
Av.	62.4	171	1.72	1903	1413	1.34	30.6 ± 2.9%	1101 ± 5.0%

[1] By DuBois formula.

It is seen that the average weight had declined 1.2 kg from the first to the second observation, thereby affecting slightly the calculated surface area (DuBois chart). The basal metabolism by the Benedict-Roth clinical apparatus, with which Mr. Anderson has had extensive experience, was quite constant in the two determinations except for one subject (G.S.). The average endogenous excretion had fallen 146 mg — a little more than the average for the eight members of the

squad (table 3). The consequence is that the coefficient, endogenous nitrogen divided by basal metabolism computed to 24 hours, also fell from 1.48 to 1.34 mg per calorie.

But the important observation is that these coefficients are much below that found by Smuts, and agree well with the ratio 1.42 reached after 30 days on no protein in Deuel's experiment.

From the final columns it is seen also that the endogenous metabolism per unit of weight is less variable than when referred to a unit of surface by the DuBois chart.

We cannot escape the impression that the alleged proportionality between a stable endogenous urinary nitrogen and basal metabolism is somewhat less than "rigorously established for all homeotherms."

SUMMARY AND CONCLUSIONS

A basic diet is described containing (with arrowroot starch) not more than 0.2 gm N for the average individual, which has been used with but slight changes over a period of nearly 20 months. This diet constituted the "no-protein" regime in 12 different periods for determination of endogenous nitrogen excretion.

It was found that the following factors influence the time within which a dependable endogenous urinary nitrogen could be obtained within 3 or 4 days, on a no-protein diet: (a) the level of protein in the pre-experimental diets; (b) the position of the no-protein period in the series of periods; (c) the nature of the protein, called here "supporting protein," immediately preceding the no-protein, and its level of intake; and (d) conditions antecedent to the supporting protein which could affect the accrued nitrogen deficit to the beginning of the no-protein period.

Dependable and stable endogenous nitrogen levels can be reached on the fourth day of no-protein, even if the nature of the supporting protein and its level of intake provide a fairly stable deposit of reserve protein, but the total antecedent conditions bring the average subject, starting fresh, to a state of accrued nitrogen deficiency equal to about 40 gm.

Stable endogenous nitrogen excretion can be reached on the third day of no-protein provided the total antecedent conditions bring the average subject to a state of accrued deficiency equal to about 60 gm nitrogen. Some evidence has been developed that subjects acquire a resistance to the depleting effect of a no-protein diet after having passed through some months of low protein diets, including five or six no-protein periods.

Blood examinations for hemoglobin and serum protein prove that members of a diet squad passing through at least 6 months subsistence on alternating high and low value proteins at a general level of not to exceed 5% of the calories can withstand at least six no-protein periods of 3 days each within that period without apparent injury to the liver, so far as its protein-forming functions are concerned.

Regression equations are given of endogenous N on body weight in 73 determinations of the urinary excretion on 28 men and in one each on seven women for the third-day no-protein diet as well as in 22 determinations on 14 men for the fourth-day no-protein. The number of observations on women is too small to derive an entirely satisfactory equation.

In duplicate observations of basal metabolism on five men at the termination of the fourth-day no-protein in two periods 1 month apart, the average coefficient, milligrams endogenous urinary nitrogen to kg-cal. of the 24-hour basal heat, was found to be 1.48 and 1.34, respectively — values which agree much more closely with those of Deuel, Sandiford, Sandiford and Boothby and with two on women reported recently by Bricker, Mitchell and Kinsman ('45) than with those of Terroine or Smuts.

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BIOLOGICAL VALUE OF PROTEINS IN RELATION TO THE ESSENTIAL AMINO ACIDS WHICH THEY CONTAIN [1]

II. INTERCONVERTIBILITY OF BIOLOGICAL VALUES ILLUSTRATED BY SUPPLEMENTING EGG AND SOY PROTEIN WITH ESSENTIAL AMINO ACIDS

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TWO FIGURES

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If two proteins of different composition are retained in different proportion because of this difference in composition, it might be expected that different effects would be obtained when the two proteins are supplemented by single amino acids added one at a time. The experimental demonstration consisted in giving to each member of the same diet squad of seven members a given amino acid in the same quantity, added first to the diet containing the egg protein at endogenous level (Murlin et al., '46), and in the next period to the diet containing the soy protein at (nearly) the same level. Table 1 shows how much of each of the seven acids was fed and the effect on biological value (B.V.) of the two proteins. Averages of the individual effects given in this table are summarized in figure 1. There was a definite increase in

[1] This study was carried out under contract with the Committee on Medical Research of the Office of Scientific Research and Development.

B.V. for only two of the seven added to egg at this level; namely, histidine and lysine, and for only one of the seven added to soy protein; namely, lysine. The latter gave the larger effect with soy, but the smaller effect with egg. It has been known since the work of Mitchell and Smuts ('32) and of Hayward, Steenbock and Bohstedt ('36) that soy protein, at least before cooking, lacks a sulfur-containing amino acid. Additional methionine to that already contained in baked defatted soy flour as in this case does not improve the B.V. The negative effects from threonine, valine and leucine, all of the racemic form, are strikingly different. Each test is the result of a 5-day experiment.

TABLE 1

Effect of individual amino acids on biological value (B.V.) of egg and soy proteins.

SUBJ.[1]	B.V. EGG ALONE	B.V. EGG + AM. ACIDS	DIF. B.V.	B.V. SOY PROT. ALONE	B.V. SOY + AM. ACIDS	DIF. B.V.	AMT. N ADDED GM	KIND OF AMINO ACID ADDED
2	85.9	85.4	− 0.5	86.1	75.6	− 10.5	0.5	*dl* Threonine
3	88.2	83.0	− 5.2	78.0	53.7	− 24.3	0.5	*dl* Valine
5	98.0	90.8	− 7.2	89.0	73.2	− 15.8	0.5	*dl* Leucine
7	90.4	91.3	+ 0.9	75.6	70.9	− 4.7	0.5	*dl* Methionine
8	101.0	93.4	− 7.6	85 3	66.2	− 19.1	0.25	*dl* Tryptophane
11	75.4	86.7	+ 11.3	75.0	55.6	− 19.4	0.25	*l* Histidine
12	95.5	99.6	+ 4.1	78.4	88.4	+ 10.0	0.25	*l* Lysine

[1] The original numbers for members of the squad are retained. Numbers 1, 4, 6 and 9 had to be excused for various valid reasons.

It is clear that adding equal quantities of the same amino acids to two different proteins eaten by the same squad gives quite different effects on B.V.; namely, an average reduction of less than 1.0 point for egg and 12 points for soy.

Figure 1 reveals some additional points of interest. The first group of columns gives the absolute biological value of egg protein. The first two show the difference between the nitrogen excretion on egg protein and on no-protein; the last two the difference for urinary nitrogen; and the middle two how the corrections for these differences are applied to food nitrogen eaten and food nitrogen absorbed, to get the percentage retained.

The next group of columns concerns the effect of superimposing a single amino acid onto the diet of each member of the squad getting egg at the endogenous level, which has just been discussed for individual effects. Now, we are concerned with the average effect, as a demonstration of reliability. Note that the average B.V. of the egg plus amino acid is 90.6% computed in the same way as the value of 92.1 is obtained, and from the same endogenous values. With these

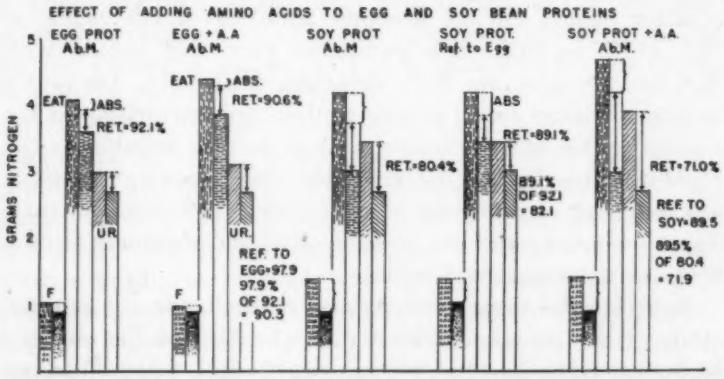

Fig. 1 These figures represent the quantities and operations necessary to obtain the biological value of a protein from two periods of feeding: (1) a no-protein diet and (2) the same diet plus the test protein. The two columns marked F represent the average excretion of a squad of seven members from the two diets in reverse order to the feeding. The last two, marked UR, represent the average excretions of urinary nitrogen from the two diets. The middle columns illustrate the two corrections to the food nitrogen. The small bracket, representing the excess of fecal nitrogen from the protein period over that from the no-protein period, corrects the protein nitrogen eaten (EAT), by the amount of waste ascribable to the food protein, to give the nitrogen absorbed (ABS.) from the protein. The two-pointed arrow, representing the excess of urinary nitrogen from the protein period over that from the no-protein period, corrects the absorbed nitrogen by the amount of waste ascribable to the absorbed food nitrogen, to give the retained (RET.) nitrogen, and this expressed as a percentage of the absorbed is the biological value. Ab. M. over a group of columns means "absolute method"; namely, estimating the B.V. of a protein against a true endogenous excretion (no-protein) level of both urinary and fecal nitrogens. Ref. to Egg means the same excretion data from a diet containing another protein, or a protein plus added amino acids, referred to the excretion data from a diet containing egg. In figure 1 the egg feeding referred to was at (or near) endogenous level; in 2 the egg feeding at 2 × endogenous level is used as base.

data available we now calculate the B.V. of the combination egg plus amino acid in terms of the excretion data on the egg protein alone. In other words, we are now using egg protein instead of body protein as the basis. The result tells us that this combination is 97.9% as good as egg protein alone.

Egg plus amino acid being 97.9% as good as egg for retention of nitrogen to replace body waste, and egg protein being 92.1% as good as the hypothetical perfect protein the combination should (in the absolute scale) be worth 97.9% of 92.1 which is 90.3. This compares very well indeed with 90.6 for the absolute B.V. determined directly. Obviously biological values based on egg protein are convertible to the absolute scale and therefore become just as reliable as the absolute value directly determinable. One needs no statistical aid to arrive at a feeling of satisfaction with this outcome. The agreement could not be so good if the fundamental data were not consistent and reliable.

Applying the same methods and reasoning to the parallel study of soy protein, we arrive at: (1) 80.4 for the absolute biological value directly determined; (2) 89.1, referred to egg as standard; and (3) 82.1 instead of 80.4 for the absolute value, indirectly determined (i.e., 89.1% of 92.1 = 82.06). This result is not quite so satisfactory.[3]

The final comparison on the chart is one involving the direct absolute B.V. for soy plus amino acids, and the value determined indirectly. Direct value is 71.0 — which of course means that adding single amino acids to a medium-value protein does not effect any improvement; rather the contrary. Referred to soy protein alone as a base, we find that the soy plus amino acid is only 89.5% as good. Soy on the absolute scale being 80.4 and soy plus the additions measuring only to 89.5% of soy, the average absolute B.V. of the combinations,

[3] If the absorption of soy protein in relation to egg had been the same as its absorption in relation to endogenous excretion (in other words, if the correction on fecal nitrogen had been the same), the converted B.V. would have been 81 instead of 82.1.

indirectly determined, is (89.5% of 80.4 =) 71.9.[3] In summary we have: for egg plus amino acids 90.6 direct, 90.3 indirect; for soy protein 80.4 direct, 82.1 indirect; and for soy plus amino acids 71.0 direct, 71.9 indirect.

Effect of level of intake of the supplemented protein

The results just described were obtained with the members of a diet squad after they had been considerably depleted of nitrogen not only by the no-protein period, but by the subsequent periods on soy protein. The supplementing effects of single essential amino acids in the amounts fed (as shown in table 1) were largely negative so far as the biological value referred to the endogenous nitrogen was concerned, and they were quite different when added to egg protein and to soy protein at the endogenous level in the same amounts. The negative effect could scarcely be due to depletion alone for this would tend to enhance retention. We must suppose that when one of the essentials is added under these conditions and produces a negative effect whatever the protein supplemented, it is not needed under the circumstances — which may be another way of saying that it is not qualitatively adapted to meet the needs of the moment.

For the purpose of testing the opposite condition, namely, repletion of protein and consequent high plus nitrogen balances, on the effects of supplementation with amino acids, the squad was next placed on a level of egg protein intake approximately double that prevailing in the above four periods, namely, 7.79 gm N or 46.8 gm protein daily (including 0.3 gm N in the remainder of the diet) instead of 4.073 gm N. The average retention for the last 3 days of this diet was 1.76 gm N daily. This reflects the influence of the soy period immediately preceding when the average balance was

[3] If the average absorption rate of soy protein plus amino acids had been the same in relation to soy protein alone, as it was in relation to the endogenous excretion, this value would have been 70.9. Actually there was a little tendency to diarrhea on this period with amino acids and the fecal nitrogen which usually is reduced when amino acids are added to a protein was increased a little. This phenomenon of reduction will be considered in a later paper of this series.

— 0.611 gm N daily. Retention of nitrogen from egg far more than made up the deficit.

When the supplementary amino acids were again added (in the next period) in equal amounts as before, there was a marked decrease in nitrogen balance in all subjects in the last 3 days of the period. Some of this decrease probably would have occurred without the amino acids, because the retention demand had been satisfied. But it is of special interest: (1) that all of the seven amino acids fed produced effects in the same direction; and (2) that this reduced retention in every case but one (leucine) exceeded the nitrogen

TABLE 2

Change in nitrogen balance when egg protein at two levels of intake is supplemented with amino acids.

(Same subjects received same amino acids in same amounts.)

GM N FROM		AT ENDOGENOUS LEVEL			AT DOUBLE ENDOGENOUS LEVEL		
		Egg alone	Egg plus am. acid	Dif.	Egg alone	Egg plus am. acid	Dif.
		gm	*gm*	*gm*	*gm*	*gm*	*gm*
0.5	*dl* Threonine	— 0.042	+ 0.139	+ 0.181	+ 1.846	+ 1.124	— 0.722
0.5	*dl* Valine	+ 0.122	+ 0.389	+ 0.267	+ 1.401	+ 0.866	— 0.535
0.5	*dl* Leucine	+ 0.350	+ 0.212	— 0.128	+ 1.504	+ 1.047	— 0.457
0.5	*dl* Methionine	— 0.217	+ 0.034	+ 0.251	+ 2.190	+ 1.409	— 0.781
0.25	*dl* Tryptophane	+ 0.652	+ 0.661	+ 0.009	+ 2.583	+ 1.762	— 0.821
0.25	*l* Histidine	— 1.483	+ 0.520	+ 2.003	+ 1.447	+ 1.016	— 0.431
0.25	*l* Lysine	— 0.004	+ 0.192	+ 0.196	+ 2.152	+ 1.795	— 0.357

contributed by the amino acid. It appears, therefore, that with the exception of a slight effect from leucine none of the amino acid nitrogen added as a supplement under these circumstances is so available for tissue repair as nitrogen from the egg protein.

Table 2 brings together for comparison the effects on nitrogen balance from supplementing with the same amounts of the seven essentials fed to the same persons, several periods apart: (1) at the endogenous level and (2) at approximately double this level. At the lower level *dl* leucine is the only one that produced a negative effect on daily nitrogen balance as

compared with the period on egg unsupplemented. At the higher level all produced a negative effect. As already noted these reduced retentions were greater than the amounts of nitrogen contributed by the several supplements, whereas in every case of supplementation at the lower level the extra

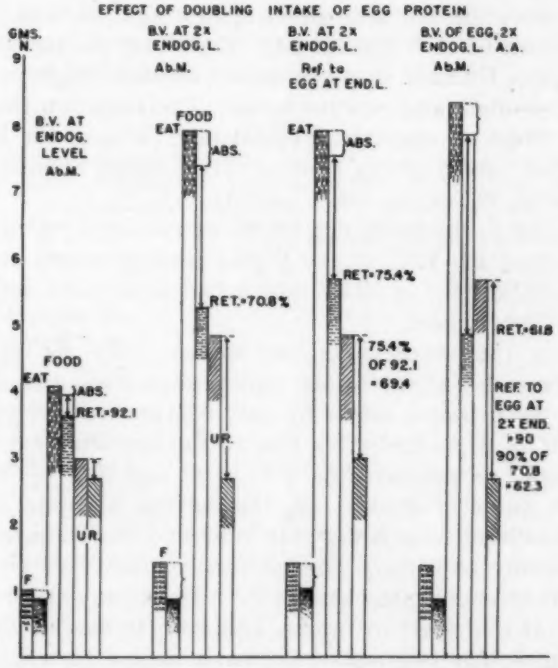

Fig. 2 The statement under figure 1 also applies to figure 2.

retention (when it occurred) was less than the amount of nitrogen contributed.

The effects of supplementation on the B.V. will be evident from the last columns of figure 2. The first group of columns on the left is the one seen in figure 1, picturing the average data obtained in a 6-day experiment on the seven subjects, for the endogenous B.V. of egg protein at an average level of

4.073 gm N. The second group gives the results on the same squad with the same basal diet, but with the egg protein at double the endogenous level. The fecal nitrogen on this higher intake is considerably elevated above the other. Comparing with the same endogenous fecal nitrogen, a larger waste is evident. However, deducting this from nitrogen ingested, the value for absorption is 96% against 97% for the endogenous level — digestibility, therefore, is not reduced materially. Urinary nitrogen shows a much larger wastage both in absolute and relative terms. The retention therefore is only 70.8% of absorbed, against 92.1. This is the B.V. of the higher intake of egg protein: 71 in round numbers compared with 92 for the lower level.[4]

Referred to the lower egg intake as standard (third group of columns) the B.V. of the higher feeding comes out 75.4, and 75.4% of 92.1 is 69.4 — not a bad agreement with 70.8, directly determined.

Adding the seven individual amino acids in the same amounts with respect to the endogenous level and thereby raising the nitrogen intake by only 0.39 gm we find an absolute B.V. of 61.8. Referring now to the egg intake at double endogenous as standard the B.V. is 90, and 90% of 70.8, the absolute value of double egg, the derived absolute, is 62.3. Thus the direct value is 61.8, the indirect 62.3. The agreement could hardly be better. The interconvertibility of biological values from endogenous base to the metabolism data from egg protein at that level of intake, and even to similar data obtained at double this intake, is clearly demonstrated.

Conversion of a relative B.V. of egg at one level against egg at another (endogenous) level by means of a conversion factor obtained on another squad

All the conversions from one base to another thus far considered were calculated from data obtained on the same squad

[4] As regards the effect of different levels of feeding the same protein on biological value, there is of course nothing new about these results. Hamilton ('39) has shown that feeding egg protein to rats at a level of 4% (of dry weight) gave a B.V. of 100; at 8% it was 91; at 12% 84; at 16% 62; and so on.

of subjects, but as shown in table 3 it can be just as readily accomplished from the general data obtained on one squad by means of a conversion factor obtained on another. This is not mere coincidence, for the same type of conversion has been obtained between other squads, as will be described in another communication.

The absolute B.V. obtained on squad 4 was referred to the endogenous nitrogens from that squad. It is lower than the value would have been if egg had been fed at a level nearer

TABLE 3

Conversion of a relative biological value (B.V.) of egg at one level of intake against egg at another (endogenous) level by means of a conversion factor obtained from experiments with another squad of human subjects.

		EGG AT 5% OF CALORIES	
		B.Vs. endogenous excretion	B.Vs. egg at endogenous level
(a)	Test protein fecal N	1.269	1.269
(b)	No-protein fecal N	1.074	1.017
(c)	Fecal waste N (a–b)	0.195	0.242
(d)	Test protein N eaten	5.798	5.798
(e)	Absorbed N (d–c)	5.603	5.556
(f)	True digestibility $(\frac{e \times 100}{d})$	95	95
(g)	Test protein urine N	3.264	3.264
(h)	No-protein urine N	2.629	2.989
(i)	Urinary waste N (g–h)	0.635	0.275
(j)	Retained N (e–i)	4.968	5.281
(k)	B.V. $(\frac{j \times 100}{e})$	88.6 Abs.	95.0 Rel. × 0.921 = 87.5 Abs.

that of the net endogenous losses (urinary and fecal nitrogens less the nitrogen in no-protein foods). When the same 5% level was evaluated against the excretion data from egg at endogenous level of feeding, the relative value was converted to absolute by the factor 0.921 obtained from squad 2. This merely illustrates the fact that the data from one squad, differing entirely in personnel and nutritional history from the other, may, if reliable, be used to interpret data from the second. Successful conversion in fact becomes a measure of reliability.

SUMMARY

1. Adding single essential amino acids to egg or soy bean protein (baked) at or near the endogenous level of intake does not improve the biological value of these proteins, rather the contrary — even though they may improve nitrogen balances.

2. Biological values of egg and soy bean proteins determined at or near endogenous levels (absolute method) may themselves be used as conversion factors to translate biological values of these proteins plus amino acids (referred to the proteins unsupplemented) to the absolute scale with very little error.

3. Likewise the absolute biological value of egg fed at double the endogenous level of intake may be used as a conversion factor to translate values relative to egg at this level to the absolute scale.

4. The conversion factor reliably determined on one squad of subjects may be applied also to the fundamental data of relative values obtained on another squad to convert the latter to the absolute scale.

5. Once these conversion factors are reliably determined they may be applied widely to relative values obtained in a proper sequence of feeding periods to bring the latter into line with absolute values, as will be shown in a later paper. Thus the existing confusion of values obtained at widely different levels of feeding can in time be completely resolved.

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THE INFLUENCE OF PHOSPHORUS, CALCIUM AND VITAMIN D_3 UPON THE FAT CONTENT OF THE SKELETON IN GROWING PIGS

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The mechanism of bone calcification has been the subject of much discussion. Most studied in this connection are the elements Ca and P, the enzyme phosphatase, and the effect vitamin D exerts upon these elements in connection with the calcification process. As to the influence of vitamin D, it appears that the earlier view that this factor acts through increased intestinal absorption of phosphorus has now been modified. New experiments with radioactive phosphorus have given results which support the theory that vitamin D exerts its effect by intensification of phosphorus turnover in bone, accompanied by hyperphosphatemia and decreased visceral phosphorus turnover.

The effect of dietary fat upon bone calcification has been the object of several studies. In his work on the so-called "anticalcifying" action of cereals, Mellanby ('22 a; '22 b; '24; '26; Green and Mellanby, '28) suggested that this effect might be related to the fatty acids in the diet. Bunkfeldt and Steenbock ('43) studied the effect of dietary fat on bone calcification in rats receiving diets completely deficient in vitamin D. They observed that when the diet had a very low content of phosphorus the calcification of the femur was diminished by fat. When phosphorus was fed at, or above, optimum levels, dietary fat increased the calcification. When the value of the Ca : P ratio was above 4 the effect was not

observed. Other workers (Jones, '43) have also reported that fat in the diet improves the absorption of calcium. Therefore, it is quite clear that the presence of fat in the diet in any case does influence the mineral absorption. Regarding the influence of the absorbed fat upon the mechanism of ossification, this has received less consideration. As to the fat in the bone, most workers are of the opinion that this merely serves as a depôt for fat storage, especially the yellow fat in hollow or long bones. The red marrow within the ends of long bones and in the interior of flat ones is known to be the place where red blood-cell formation takes place (Carlson and Johnson, '37). It is also known, that in the severely anemic patient the active bone marrow is always increased in amount and may extend throughout the long bones (Minot and Strauss, '45). Hirsch and Weinhouse ('43) have reported a study of atherosclerosis, and this process showed a great similarity to normal bone formation. In their study Hirsch and Weinhouse summarized that the simple fatty deposits in atherosclerosis had the same lipid composition as blood plasma and the normal intima, and that after the lipids are deposited and remain in the tissue, the subsequent stages of atherosclerosis follow.

Against this incomplete picture of the lipid rôle in ossification, it is of interest to study how the lipid content of bone is influenced by certain factors in the food, such as mineral nutrients and vitamin D. In the experiments reported in this paper the influence of minerals and of vitamin D upon the fat content of the bone has been studied.

INFLUENCE OF PHOSPHORUS AND CALCIUM UPON THE FAT CONTENT IN THE BONES

The experiments were planned for the study of the influence of the diet upon the calcification process in growing pigs; several series of experiments were carried out. All the pigs used were of the Swedish Landrace breed. The diet in the experiment consisted of 50% ground corn and 50% ground oats and an average of 2.5 kg of skimmed milk per animal

daily. In order to satisfy the mineral requirement, from 20 to 40 gm of limestone and 5 to 10 gm of common salt (NaCl) were added daily. Vitamin A was provided through the carotenoids in the yellow corn; extra vitamin D was not given. The average percentage composition of this diet figured on the basis of dry matter, was the following: Crude fiber 6.5, nitrogen-free extract 68.6, crude protein 12.0, crude fat 3.8, calcium (Ca) 0.82, and phosphorus (P) 0.42. The analyses of the feed for these components were carried out by standard methods, in agreement with the regulation given by the Swedish Royal Board of Agriculture ('40).

The first experiment included 16 pigs divided as equally as possible into two groups, 1 and 2. The animals were divided according to litter, sex, weight and pre-experimental development. The experimental feeding began when the animals weighed 34 kg and finished at a weight of 100 kg, when the animals were slaughtered. During the period of the experiment, which covered more than 3 months, group 1 received the diet and the mineral supplements mentioned. The Ca : P ratio was 1.95. Group 2 received in addition 40–45 gm of di-sodium phosphate per pig daily. The supply of Ca and P for this group averaged 0.81% of Ca, and 0.58% of P calculated on the dry matter with a Ca : P ratio of 1.40. The development of the pigs during the experiment was controlled by weighing the animals at weekly intervals and by controlling the feed consumption. The daily average weight-increase in group one was 658 gm and in group two 719 gm. After slaughter one femur was taken out, prepared and analyzed for dry matter, crude fat and ash. The fat was determined according to the method of Schmid-Bondzynski-Ratzlaff ('03). The bones were prepared before the analysis in such a way that they were freed as far as possible from soft tissue such as fat, muscles and connective tissue by scraping the surfaces with a knife. After this the large bone was first crushed in a large meat-grinder (the Huskvarna "fox mill" no. 8), whereupon the material was homogenized in a special laboratory grinder. This method of preparation explains the rela-

tively high values of fat content in the bone. The method was used because of the large amount of material prepared. As is seen from the data given, this method gave, however, satisfactory results. The results of the analyses are summarized in table 1.

From the results given in table 1 it may be seen that under the conditions described extra phosphorus in the diet decreases the amount of fat in the bones. This effect, however, is not specific for phosphorus alone.

TABLE 1

Fat content of the femur as affected by supplementing the diet with phosphorus and by vitamin D_2.

EXPERIMENT NUMBER AND GROUP	NUMBER OF ANIMALS	REGIMEN	FEED (per kg dry matter) in grain and milk		supplements		FEMUR[1] crude fat (dry basis)	FEMUR[1] ash (dry basis)
			Ca	P	Ca	P		
			gm	*gm*	*gm*	*gm*		
VII		With and without supplements of phosphorus[2]						
1	10	Without extra P	2.5	4.2	5.7	..	36.9 ± 0.85	62.7 ± 0.71
2	10	With extra P	2.5	4.2	5.6	1.6	32.1 ± 1.75	60.3 ± 1.46
IV		With and without supply of vitamin D_2[3]						
1 a	4	With D_2	1.8	4.0	..	..	41.9 ± 0.92	59.5 ± 0.53
1 b	4	Without D_2	1.8	4.0	..	..	37.5 ± 1.01	59.5 ± 0.24
2 a	4	With D_2	1.8	4.0	2.4	..	40.2 ± 0.72	61.2 ± 0.29
2 b	4	Without D_2	1.8	4.0	2.4	..	34.0 ± 2.77	61.2 ± 0.51
3 a	4	With D_2	1.8	4.0	5.4	..	38.5 ± 1.37	60.8 ± 0.95
3 b	4	Without D_2	1.8	4.0	5.4	..	37.2 ± 1.32	60.7 ± 0.61
4 a	4	With D_2	1.8	4.0	7.1	..	38.4 ± 1.01	61.8 ± 1.07
4 b	4	Without D_2	1.8	4.0	7.1	..	36.5 ± 1.31	61.9 ± 1.05

[1] Values are given with their standard errors calculated from $\Sigma = \frac{\sigma}{\sqrt{n}}$ where $\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$, "d" is the deviation from the average, and "n" the number of observations.

[2] The basal diet consisted of 50% ground corn, 50% ground oats and an average of 2.5 kg skimmed milk per animal daily; 20 to 40 gm limestone, and 5 to 10 gm common salt daily. Ca/P value was 1.95. Group 1 received the basal diet only; group 2 received in addition 40–45 gm Na_2HPO_4 per pig daily.

[3] The basal diet consisted of 75% oatmeal, 25% corn meal, and 2.5 kg skimmed milk per animal daily. Group 1 received no extra minerals except NaCl; other groups received Ca in form of limestone. Ca/P values were: for group 1, 0.45; 2, 1.05; 3, 1.80; 4, 2.23. D_2 was provided in form of a concentrate called "Delta" which contained 200 I.U. per gm.

From feeding experiments made in Denmark in which minerals were fed to pigs Petersen ('43) has reported data for Ca similar to those given above for P; however, this effect of Ca or P is not mentioned in Petersen's paper. In his report Petersen has given the content of crude fat in the scapulae of pigs, fed on a diet of grain and skimmed milk with and without any addition of limestone. The diet contained 2.3 gm Ca and 4.8 gm P per kg of dry matter of the feed. In one experiment this diet gave a fat content of 14.4%, figured on the basis of dry matter in the bone. When 1.7 gm Ca was given as a supplement per animal daily, the fat content was lowered to 12.5%. In another similar experiment the fat content of the bone was lowered from 17.1% to 12.2%, when 3 gm Ca was supplied in the diet. In another case the supplement was raised to 9 gm Ca, and this reduced the fat content to 9.2 gm. The data cited represent the average values for 7 to 10 experimental animals. It should be noted that the bone investigated in this case was a flat bone containing red marrow. Evidently the observation holds true for red as well as for yellow bone-marrow fat.

Since it has been shown that phosphorus alone or calcium alone exerts the influence mentioned upon the content of bone-marrow fat, it might be expected that these two elements, when given together as supplements in the feed should likewise reduce the amount of crude fat of the bone. That such is the case is also indicated by Petersen's data. When no extra minerals were supplied in the diet mentioned, the fat content of the scapulae was 14.0% figured on basis of dry matter. When 6.4% Ca and 2.3 gm P were given daily as supplements to the feed, then the fat content in the bone in one experiment was only 10.3%. In another experiment 16.1% fat in the bone was obtained when no minerals were supplied. When 5.5 gm Ca and 1.0 gm P were supplied as minerals, 11.3% fat was obtained as average for the bone. Each value is based upon the results from 6 to 8 experimental animals. The Ca and P were added in the form of commercial dicalcium phosphate or bone meal.

INFLUENCE OF VITAMIN D UPON THE FAT CONTENT IN THE BONES

A series of experiments was carried out where the effect of vitamin D upon the fat content in the bone could be observed. The feed used during the experimental period consisted of 75% of oatmeal, 25% of corn meal, and 2.5 kg of skimmed milk per animal daily. Thirty-two pigs were used in the experiment; the animals were divided into four groups, each group containing 8 animals. The experiment began when the animals weighed about 45 kg and was continued until they weighed 125 kg, a period of about 4 months. Group 1 received no extra minerals except common salt; Ca in percent of dry matter in the feed averaged 0.18 and P 0.40. The other groups received extra Ca in the form of limestone, which increased the Ca-content of the feed as shown in table 1. The values for the Ca : P ratio were: group 1, 0.45; 2, 1.05; 3, 1.80; and group 4, 2.23.

Each group was divided into two subgroups including 4 pigs, where one subgroup received no supplement of vitamin D, and the other received a supplement of 1 gm of a preparation of D_3 containing 200 I.U. per gm. Consequently, 16 animals in the experiment received extra vitamin D, and 16 animals obtained no such supplement. In addition to other observations made during and after the experiment, the femur was analyzed for dry matter, ash and fat. The results of these analyses are given in table 1.

The results indicate that vitamin D_3 in its influence upon the fat content in bone has an effect opposite to that demonstrated for Ca and P. Thus, the fat content in the bones of all the groups reported in table 1 is higher when vitamin D is added to the diet than when no such vitamin is added. It is of interest to observe, however, that the difference in fat content between groups fed with and without vitamin D is much more pronounced where only small amounts or no supplements at all of Ca are given than it is when relatively large amounts of Ca are given. In groups 3 and 4, where 5 and 7 gm of Ca are given as supplements per kg of dry matter, the

difference in fat content in the bone is only 1.3 and 1.9%, while the corresponding figures in groups 1 and 2, which received only 2.4 gm or no Ca at all as supplements, are 4.4 and 6.2%. It appears therefore that, as far as the fat content of the bone is concerned, the influence of vitamin D has been counteracted by the influence of the extra Ca given to groups 3 and 4, so that in this case the special effect of vitamin D_3 is relatively small.

SUMMARY

1. Feeding experiments with pigs were carried out where the diet consisted of grain, such as barley, oats, and corn, and, in addition, 2.5 kg of skimmed milk daily per animal was given. In some cases Ca was added to the diet, in other cases P, and in other instances vitamin D_3. The experimental period included a time of 3 months or more, and continued from a weight of the animals of 35–45 kg up to 100 kg or more. Among other observations which were carried out in connection with the experiment, the fat content of a bone of the skeleton of the animals was studied.

2. Calcium, or phosphorus, or both elements together, decreased the content of fat in the femur when the elements were added as supplements to the diet mentioned. This observation of ours made on a long, hollow bone, such as the femur, agrees with that made by Petersen on a flat bone, the scapulae.

3. Vitamin D_3 appeared to increase the amount of fat in the femur when this vitamin was given to the pigs under the conditions described. When other Ca or P was given simultaneously as a supplement to the diet the increase in fat content in the bone was less pronounced. It therefore appears as if Ca and P on the one hand and vitamin D_3 on the other counteract each other in this influence upon the fat content of the skeleton.

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THE ASCORBIC ACID CONTENT OF WHOLE BLOOD PLASMA OF NORMAL RATS WITH EVIDENCE OF A SEX DIFFERENCE [1]

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An answer to the question of what is a "normal" level of ascorbic acid in human blood would aid in the determination of nutritional status. Although plasma values of zero have been reported without clinical signs of scurvy, there must be some blood level of ascorbic acid at which the body functions best, and values below that level might indicate malnutrition with regard to ascorbic acid.

A number of investigators have reported that values below 0.7 mg ascorbic acid per 100 ml of plasma indicate inadequate dietary intakes of ascorbic acid. Munsell et al. ('44) have raised the question of what, if any, level of plasma may be taken as indicative of vitamin C deficiency since determinations made in that laboratory on healthy animals receiving an adequate diet showed plasma values of 0.25 mg to 0.56 mg. Some plasma values reported for a number of different animals by various investigators are shown in table 1. These values are from apparently normal healthy animals receiving adequate diets. With the exception of the low values for the dog and high values for hens those for the other animals lie between 0.30 mg and 0.66 mg.

Rats synthesize ascorbic acid; therefore it might be assumed that plasma values of healthy rats maintained on an adequate diet would be an indication of normal values, at

[1] This investigation was made possible by grants from the General Education Board and the University of Alabama Research Fund.

least for that species. This investigation was undertaken to determine the level of ascorbic acid in the plasma of adult rats and since several investigators have suggested that the values for whole blood are more reliable than plasma values, deter-

TABLE 1

Plasma ascorbic acid values for different animals as reported in the literature.

ANIMAL	PLASMA ASCORBIC ACID	REFERENCE
	mg/100 ml	
Monkey	0.41	Munsell et al. ('44)
Guinea pig	0.56	Munsell et al. ('44)
Guinea pig (10)[1]	0.54	Todhunter and Brewer ('40)
Guinea pig	0.30	Karel and Chapman ('44)
Rabbit	0.41	Munsell et al. ('44)
Goat	0.46	Munsell et al. ('44)
Horse	0.46	Munsell et al. ('44)
Mare	0.53 ± .17	Rasmussen et al. ('44 a)
Sheep	0.56	Rasmussen et al. ('44 b)
Lambs	0.48 ± .21	Rasmussen et al. ('44 b)
Ewes (20)	0.66 (0.43–0.82)	Satterfield et al. ('42)
Dairy calves (19)	0.32 (0.03–0.77)	Bortree et al. ('42)
Dairy heifers (4)	0.49 (0.24–0.80)	Bortree et al. ('42)
Dairy cows (24)	0.44 (0.11–0.80)	Bortree et al. ('42)
Hens, 12 weeks age	2.05 (1.46–2.43)	Satterfield et al. ('45)
Dog	0.25	Munsell et al. ('44)
Dog, 2 mo.–6 yrs. (37 ♂)	0.353 (0.172–0.840)	LaCroix et al. ('42)
Dog, 2 mo.–6 yrs. (19 ♀)	0.368 (0.126–0.743)	LaCroix et al. ('42)
Dog, 6–14 yrs. (24 ♂)	0.353 (0.168–0.591)	LaCroix et al. ('42)
Dog, 6–14 yrs. (17 ♀)	0.308 (0.118–0.620)	LaCroix et al. ('42)

[1] Number of animals shown in brackets.

minations on whole blood were also made. The hemoglobin content was measured at the same time to see whether there was any relation between hemoglobin and ascorbic acid content of the blood.

EXPERIMENTAL

Animals

Albino rats from the breeding colony, raised on a dog chow,[2] supplemented once a week with lean meat or liver, and

[2] Purina Dog Chow Checkers.

lettuce or carrot, were used. The first group of animals were mature breeders; later, several litters were raised specifically for this study and were sacrificed at 250 gm weight for males and 200 gm for females.

Animals were anesthetized by intraperitoneal injection of a solution of 20 mg nembutal (Kuether, Telford and Roe, '44) and within 15 to 20 minutes an incision was made and the heart exposed. Approximately 5 ml of blood were withdrawn in a syringe and used for hemoglobin and ascorbic acid determinations.

Hemoglobin

Hemoglobin determinations were made with a photelometer [3] according to the method of Sheard and Sanford ('29). For each determination two aliquots of 0.02 ml of blood were used. The accuracy of the method was verified by comparison with results obtained by the oxygen capacity method of Van Slyke [4] (Van Slyke and Neill, '24).

Ascorbic acid

Plasma ascorbic acid was determined by a modification of the micromethod of Mindlin and Butler ('38), omitting the use of KCN. In order to obtain the necessary volume for reading in the Coleman spectrophotometer 0.5 ml plasma filtrate and 1 ml buffered dye were used. Sodium-acetate buffer was used in such concentration (2.25%) to give a pH of 4.1 for the dye-plasma filtrate mixture; pH measurements were made with a Beckman pH meter. Corrections were made for turbidity. The addition of pure ascorbic acid to plasma analyzed by this procedure gave recoveries of 96.6 to 110.3%, which compare favorably with those reported in the literature (Mindlin and Butler, '38).

The method of Roe and Kuether ('43) was used to determine the ascorbic acid content of the whole blood, using 2 ml

[3] Cenco-Sheard-Sanford, Type C-5.

[4] We are indebted to Dr. J. M. Bruhn of the University of Alabama Medical College for making this determination.

of blood for each determination. Recoveries of added ascorbic acid by this method were 89.3 to 100.9%.

RESULTS

Hemoglobin values are shown in table 2; there was no correlation between this compound and the ascorbic acid content of plasma or whole blood. The difference in hemoglobin value for the two sexes was statistically significant.

TABLE 2

Mean ascorbic acid and hemoglobin values of blood of rats grouped according to body weight and sex. Range and standard error are included.

BODY WEIGHT	HEMOGLOBIN	ASCORBIC ACID	
		Plasma	Whole blood
gm	*gm/100 ml*	*mg/100 ml*	*mg/100 ml*
Males			
250–299 (23)	13.6 ± 0.14 (12.0–14.7)	0.84 ± 0.052 (0.33–1.36)	0.70 ± 0.026 (0.49–0.91)
300–450 (27)	13.8 ± 0.13 (12.8–16.1)	0.90 ± 0.026 (0.62–1.21)	0.74 ± 0.022 (0.55–0.99)
Mean (50)	13.7 ± 0.10	0.87 ± 0.028	0.72 ± 0.017
Females			
200–249 (26)	13.0 ± 0.11 (11.9–14.1)	0.33 ± 0.029 (0.01–0.63)	0.34 ± 0.011 (0.23–0.46)
250–350 (25)	13.0 ± 0.21 (11.2–14.8)	0.33 ± 0.024 (0.09–0.61)	0.36 ± 0.019 (0.23–0.65)
Mean (51)	13.0 ± 0.12	0.33 ± 0.019	0.35 ± 0.011

The values for ascorbic acid in whole blood and plasma for both sexes are given in table 2. The males had a mean value of 0.87 ± .028 mg per 100 ml of plasma, with a range of 0.33 to 1.36 mg; the mean value for whole blood was 0.72 ± .017 mg, with a range of 0.49 to 0.99 mg. The corresponding values for females were 0.33 ± 0.019 mg with a range of 0.01 to 0.63 mg for plasma, and 0.35 ± 0.011 mg with a range of 0.23 to 0.65 mg for whole blood.

There was no correlation for either sex between the ascorbic acid in the plasma or whole blood and the age or weight of the adult animals.

There was a highly significant difference between the two sexes in ascorbic acid content of both whole blood ($0.37 \pm .020$ mg) and plasma ($0.54 \pm .033$ mg). The reason for this difference is not apparent. It is possible that the sexes have equal ability to synthesize ascorbic acid but that utilization is greater on the part of the female and thus brings about lower blood values. Sutton et al. ('42) reported ascorbic acid values of 0.588 mg to 0.895 mg per 100 mg of plasma for male rats receiving varying levels of vitamin A as supplements to the diet. No plasma values were given for females but there was found to be a sex difference in the excretion of ascorbic acid. Female rats excreted only about half as much ascorbic acid as did the males.

An interesting relationship was shown between the ascorbic acid content of the plasma and whole blood (table 3). At the lower levels of plasma ascorbic acid, 0.01 to 0.60 mg for males and 0.01 to 0.30 mg for females, the values for ascorbic acid per 100 ml of whole blood were higher than the plasma values, but from that point, at any plasma level the value for whole blood was lower than the plasma value.

Butler and Cushman ('40) using different methods of analysis have reported that at low levels, or even when the plasma ascorbic acid has become zero there are still measurable amounts of ascorbic acid in the whole blood. In this connection it is of interest to note that in the present study the males, whose mean whole blood ascorbic acid level was 0.83 mg, showed a highly significant difference of 0.15 ± 0.032 mg between whole blood and plasma levels. In contrast, the females, with the definitely lower mean whole blood value of 0.35 mg, did not have a significantly different plasma level. These data support the view that ascorbic acid enters the red cells with difficulty and tends to remain in the cells when the plasma level falls.

If it can be assumed that the blood plasma levels for all mammals are similar (table 1) and that the values obtained for mature rats which synthesize their ascorbic acid are indicative also of normal levels for humans then plasma values currently reported as desirable for humans are too high, at least for females.

The data of the present report also indicate the desirability of studying the sexes separately in ascorbic acid investigations.

TABLE 3

Difference between whole blood and plasma ascorbic acid at various plasma levels for albino rats.

PLASMA ASCORBIC ACID	MALE		FEMALE		MALE AND FEMALE	
	Number	Whole blood minus plasma (average)	Number	Whole blood minus plasma (average)	Number	Whole blood minus plasma (average)
mg/100 ml		*mg*		*mg*		*mg*
0.01–0.09			3	+ .243	3	+ .243
0.10–0.19			5	+ .140	5	+ .140
0.20–0.29			10	+ .054	10	+ .054
0.30–0.39	2	+ .215	17	− .003	19	+ .020
0.40–0.49			12	− .050	12	− .050
0.50–0.59	1	+ .020	2	− .125	3	− .079
0.60–0.69	4	− .050	2	− .100	6	− .066
0.70–0.79	8	− .100			8	− .100
0.80–0.89	13	− .128			13	− .128
0.90–0.99	8	− .195			8	− .195
1.00–1.09	10	− .218			10	− .218
1.10–1.19	1	− .320			1	− .320
1.20–1.29	2	− .440			2	− .440
1.30–1.39	1	− .450			1	− .450

SUMMARY

Mature, normal rats, 50 males and 51 females, were sacrificed for determination of the ascorbic acid content of the plasma and whole blood, and of hemoglobin values.

The ascorbic acid content of the plasma of males was 0.87 ± .028 mg, and of females was 0.33 ± .019 mg per 100 ml; the difference was highly significant.

The ascorbic acid content of the whole blood of males was $0.72 \pm .017$ mg and of females was $0.35 \pm .011$ mg per 100 ml; the difference again was highly significant.

There was a significant difference in hemoglobin values for the two sexes, 13.7 ± 0.10 gm per 100 ml for males and 13.0 ± 0.12 gm for females. There was no correlation between the hemoglobin and the ascorbic acid content of the blood nor between either of these substances and the weight or age of the adult animals.

At low levels of plasma ascorbic acid the corresponding values for ascorbic acid in whole blood were higher; at plasma levels of 0.59 mg for males and 0.30 mg for females the order of the values was reversed.

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THE RELATIVE EFFICIENCY OF DIFFERENT FORMS OF INTRAVENOUSLY ADMINISTERED NITROGEN ON NITROGEN BALANCE AND AMINO ACID EXCRETION[1]

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FIVE FIGURES

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Methods for determining the relative nutritive values of proteins have been in use for many years. These involve principally measurement of the rate of growth, gain in weight per gram of nitrogen ingested, nitrogen exchange and balance, reproduction, and plasma protein and hemoglobin regeneration (Mitchell and Hamilton, '29). The recent availability of protein hydrolysates and crystalline amino acids suitable for parenteral injection raises the question of the relative values of such substances for nitrogen retention when they are given by vein. Since the report of Henriques and Andersen in 1913 it has been repeatedly shown that protein hydrolysates can be utilized by the body when given intravenously (Elman and Weiner, '39; Shohl et al., '39; Farr and MacFayden, '39; Elman, '40). However, the efficiency with which such materials effect nitrogen retention when given by vein is not known. Such factors as the unnatural isomers, deleterious changes during manufacture or processing, varying rates of injection and the renal excretion of amino acids and

[1] Presented before the Society for Pediatric Research, Atlantic City, September 25–26, 1944.

peptides, may conceivably interfere with the utilization of forms of nitrogen which are satisfactory when orally ingested.

A limited number of observations comparing oral and parenteral administration or comparing various nitrogenous substances when parenterally administered have been made. Elman et al. ('42), showed that an enzymic casein hydrolysate was as effective in plasma protein regeneration when intravenously administered as when it was given orally. Cox and Mueller ('44 b) compared enzymic hydrolysates of three proteins, casein, lactalbumin and plasma protein, when both intravenously and orally administered. Madden et al. ('44) compared mixtures of crystalline amino acids with various protein hydrolysates administered parenterally as well as orally to anemic and hypoproteinemic dogs, and a recent report from the same laboratory indicates that intravenous administration is not as effective for plasma protein production as the oral route (Madden et al., '45 a). Albright ('45) has found oral or intravenous administration of a casein hydrolysate equally effective in maintaining nitrogen balance.

To study the efficiency of nitrogen retention, three dogs were depleted of their nitrogen stores by feeding a diet adequate in calories but low in protein for a protracted period. Subsequently, during periods of not less than 10 days each an amount of nitrogen calculated to just equal the average daily nitrogen loss for the last 17 days of the depletion period was injected intravenously. With this low level it was expected that too little nitrogen would be retained to affect the nitrogen deficit, and that the dog would remain suitable for comparative assay purposes. In actual fact there was usually nitrogen equilibrium or a slight nitrogen loss during the first assay period so that the nitrogen need of the animal was unchanged.

Three sources of nitrogen were employed: a pancreatic hydrolysate of casein [2]; a mixture of ten essential amino acids and glycine (Mixture V_u of Madden [3]); and a mixture of ten essential and ten unessential amino acids which we calculated

[2] Amigen.

[3] The composition of this mixture was given to us by Dr. Madden.

as representing the approximate composition of casein (referred to as Mixture XII). Each was administered intravenously for a total of 10 days in 6 to 10% solution (as determined by solubility) at the same nitrogen level. When studies on these low levels had been completed, it seemed also desirable to compare retentions at higher nitrogen levels. Accordingly the minimal nitrogen level was doubled and the nitrogen balances for periods of 10 days again compared. The casein hydrolysate and mixture V_u were then compared at nitrogen injection levels which were four and eight times the minimum calculated for equilibrium.

At the lowest level of nitrogen the mixture of ten essential amino acids and glycine was most efficient in promoting retention; at the doubled level this mixture and the hydrolysate were about equally effective, and at the quadrupled and octupled levels, the hydrolysate effected much larger retention than the crystalline amino acid mixture. The mixture of amino acids calculated to represent the composition of casein was considerably less efficient in retention than the other two preparations.

EXPERIMENTAL

The three dogs selected for this study had been previously maintained in metabolism cages on various levels of nitrogen for about 100 days. During a portion of the time they had received intravenous injections of amino acid mixtures and the casein hydrolysate in a study unrelated to the present one. The basal diet consisted of a mixture of the Weech-Goettsch ('38) low protein diet and a commercial preparation known as Gaines' dog food. It was supplemented by the daily administration to each dog of 3 mg thiamine, 6 mg riboflavin, 30 mg niacin and 5 drops Oleum Percomorphum since in earlier studies signs of vitamin B deficiency had been observed when the dogs subsisted for protracted periods on the Weech-Goettsch diet. The essential nutritive histories are as follows:

Dog 1 ("Nig"), a male mongrel hound, received 0.25 gm nitrogen and 40–70 cal. per kg body weight daily in his basal

diet for 110 days; and at intervals, intravenous injections of various forms of nitrogen. At the end of this period he weighed 57 pounds and was in good nutritive condition. Dog no. 2 ("Police"), a male police dog, weighed 49 pounds when brought from the kennels and placed on metabolism. During the preliminary 110-day period he was fed 0.44 gm of nitrogen and 40 cal. per kg body weight, and also received certain intravenous injections of nitrogen. He weighed 44 pounds at the end of the 110-day period, and was in excellent condition. Dog no. 3 ("Part Police") a male mongrel police, weighed 42.5 pounds in the kennels when his food was reduced to near starvation — 8 cal. and 0.1 gm nitrogen per kilo, daily for 15 days. For the next 27 days he was fed 43 cal. and 0.12 gm of nitrogen per kilogram. During these 42 days he lost 10.5 pounds and at the end was in very poor nutritive condition. Supplementation with various levels of intravenously administered nitrogen during the next 63 days restored his weight to 36.5 pounds, at which time the present study was begun.

Since the early report of Cowgill ('28) it has been customary to feed dogs 80 cal. per kg body weight. This amount is based on the ad libitum food consumption of dogs allowed a moderate amount of exercise. Since Benedict ('38) has shown that the basal heat requirement of dogs is approximately 35 cal. per kg, it has been our custom to supply 50 cal. per kg to dogs confined in metabolism cages. In our experience this has been a satisfactory energy level. Collections of urine (preserved with thymol) and feces were made daily, and combined to make periods of 3 or 4 days. The dogs were not catheterized.

Depletion period. At the start of the study, the three dogs were fed a mixture of the two diets mentioned above calculated to supply progressively smaller amounts of nitrogen and a constant caloric intake. The minimum daily nitrogen intake was .04 gm per kilo for from 17 to 24 days. The depletion period lasted 56 days. The average daily loss during the last 17 days was .099, .057 and .091 gm nitrogen per kg body weight

for Dogs 1, 2 and 3, respectively. They lost, respectively, a total of 130, 82 and 57 gm of nitrogen, and in body weight 3.5, 1.6 and 2.5 kg, during the whole depletion period.

Supplementation materials. The particular lot of casein hydrolysate (10,014–15) employed in this work was not as adequate for nutrition as most manufactured batches (Mueller et al., '40). The total nitrogen was 12.4%, the amino nitrogen 7.7% and the amino nitrogen after acid hydrolysis, 10.5%. The average gain in weight per day of 10 litter-mate rats maintained for 8 weeks on 3 different levels of this lot as the

TABLE 1

Per cent composition of amino acid mixtures.

	Mixture V_u [1]	*Mixture XII* [2]
dl-threonine	7.0	2.0
dl-valine	11.0	12.6
dl-leucine	18.0	7.1
dl-isoleucine	12.0	6.3
l(+) lysine HCl	12.0	9.1
dl-tryptophane [3]	4.0	1.8
dl-phenylalanine	12.0	6.2
dl-methionine	6.0	2.8
l(+) histidine HCl	4.0	2.7
l(+) arginine HCl	7.0	5.0
Glycine	7.0	2.4
dl-alanine		3.0
dl-norleucine		2.0
l(−) proline		6.3
l(−) hydroxyproline		1.6
l(+) glutamic acid		17.4
dl-aspartic acid		3.2
dl-serine		2.4
l-tyrosine		5.1
l-cystine		1.0

[1] Composition kindly furnished by Dr. S. C. Madden.

[2] The analytical values which we chose for this mixture were too low in threonine and leucine, and should have been doubled since the unnatural forms are not available for growth. This quantitative deficiency accounts for the observed lower nitrogen retention. $NaHCO_3$, 9.2 gm per 100 gm of the amino acid mixture was added just before making the solution.

[3] Mixture V_u was designed to supply 4.0% l-tryptophane. Since we had only the dl-form available, this was used at the same level as there is evidence that the unnatural isomer is utilized; and even if it is not, 2% of the natural isomer is probably adequate.

sole source of nitrogen was: 1.2% nitrogen, 1.14 gm daily gain, 1.7% nitrogen, 1.45 gm gain; 2.4% nitrogen, 1.67 gm gain. It was made in 10% solution, sterilized by Seitz filtration and given intravenously by slow drip. The composition of the two mixtures of crystalline amino acids is given in table 1. Limited by solubilities, these were made in 7% solution and sterilized by autoclaving. Mixture XII deposited crystals, presumably tyrosine, on standing. These were filtered off, and a nitrogen determination made on the filtered solution in order to calculate the amount to be injected.

TABLE 2

Comparative amounts of essential amino acids supplied by the three supplements.

AMINO ACID	GM/DAY SUPPLIED ONE DOG BY LOWEST AMIGEN LEVEL	MULTIPLES OF AMIGEN SUPPLIED BY	
		Mixture XII	Mixture V_u
Threonine	0.57	0.66	2.16
Valine	0.97	2.44	1.98
Leucine	1.76	0.76	1.79
Isoleucine	0.90	1.32	2.34
Lysine	0.97	1.76	1.82
Tryptophane	0.26	1.32	2.73
Phenylalanine	0.75	1.55	2.79
Methionine	0.49	1.06	2.12
Histidine	0.37	1.38	1.91
Arginine	0 62	1.51	1.97
Gm essential amino acids daily	7.65	10.43	16.23
Gm mixture daily	18.29	18.79	17.45

These three materials necessarily differed in their content of the essential amino acids. As an approximation only, the amounts of the essential amino acids supplied by the hydrolysate, by Mixture V_u and by Mixture XII are compared in table 2. The amount of these acids present in the hydrolysate was determined by calculation from the amino acid composition of casein and pancreas as given by Block and Bolling ('45) and was used as the basis of comparison. The values (in grams of the hydrolysate) are based on the weight of

material supplied to one dog, and the values in the other two columns express the relative amount of each amino acid contained in the mixture when it was given at the same nitrogen level. This comparison shows Mixture XII (since it was based on early values for the composition of casein) to contain less threonine and leucine than the calculated amount in the hydrolysate. Since the unnatural isomers of certain amino acids are probably not used in metabolism (Gilman, '43), the mixture may also be relatively low in the active form of isoleucine. All of the amino acids contained in the enzymic hydrolysate are probably in the active form. Mixture V_a contained about twice as much of each essential amino acid as contained in the hydrolysate, and this quantity would allow for the unnatural enantiomorphs.

This comparison must be interpreted with caution, because the composition of the hydrolysate is calculated, and no correction for the optical forms of the pure acids is made. An assay of a sample of the hydrolysate using available techniques [4] showed values lower than those calculated for certain acids, and higher values for others. The specific values, calculated and determined are, respectively: leucine 9.6, 8.0; methionine 2.7, 2.0; arginine 3.4, 4.9; phenylalanine 4.1, 4.0; isoleucine 4.9, 6.0; valine 5.3, 5.6; tryptophane 1.0, 0.6%.

Administration. All infusions were given by slow intravenous drip in the radial veins while the dogs were suspended in a Pavlov frame with legs dependent. The rate was adjusted to the tolerance of the dog, so as to prevent nausea and vomiting.

RESULTS

The data are presented graphically in figures 1, 2 and 3. The values for caloric intake and nitrogen intake are plotted per kg body weight. Nitrogen balance, urinary and fecal nitrogen, weight, plasma albumin and total plasma protein and the hematocrit values are plotted in absolute quantities.

[4] Values determined for us by William T. Thompson Co., Los Angeles, using a microbiological assay procedure developed by Shankman et al. ('43).

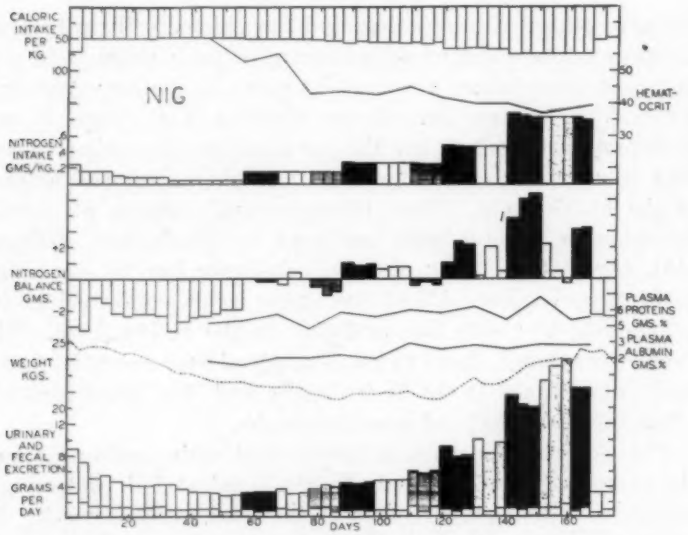

Figure 1

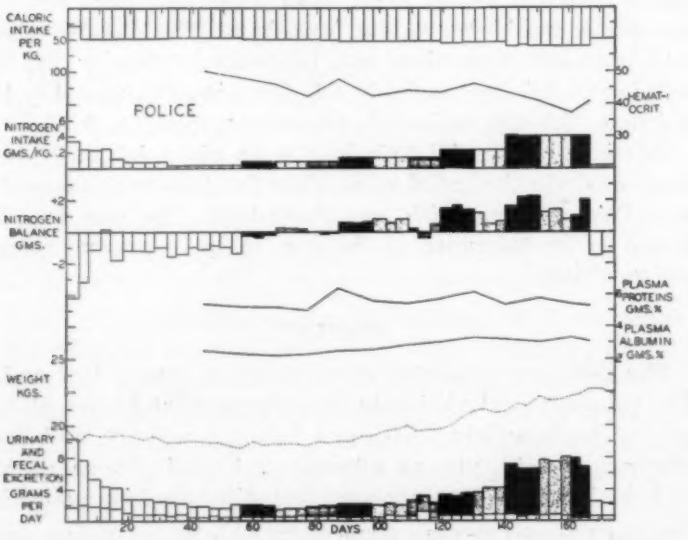

Figure 2

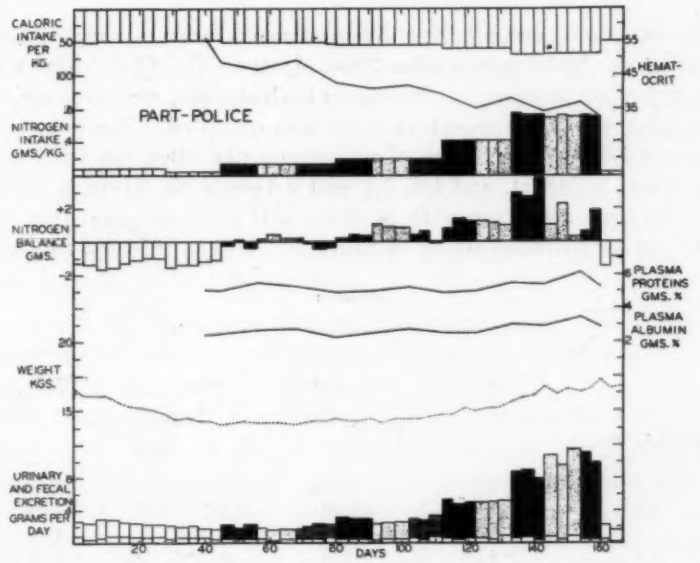

Fig. 3 Graphic record of Dog 3, constructed as described for figure 1. With this dog the nitrogen retention with mixture XII equalled that with the hydrolysate.

Nitrogen balance. At the lowest level, each of the 3 dogs retained the V_u mixture of amino acids slightly more efficiently than the casein hydrolysate. Dogs 2 and 3 showed the most pronounced difference in retention while Dog 1 showed but little. At the doubled levels the average nitrogen balances for Dogs 2 and 3 again showed a higher retention with Mixture V_u, although Dog 1 retained the casein hydrolysate most efficiently at this level. At the quadrupled levels the situation

Fig. 1 Graphic record of Dog 1 during protein depletion and the subsequent intravenous injection of 3 sources of nitrogen at four intake levels. The injections of enzymic hydrolysate (Amigen) are indicated by the solid black columns; the ten crystalline essential amino acids and glycine (V_u) by the dotted columns, and a mixture of ten essential and ten unessential amino acids (XII) by the horizontally-ruled columns. Each indicated period covers 3 or 4 days, and each supplement was administered for at least 10 days. Urinary and fecal nitrogen excretion are separately indicated.

Fig. 2 Graphic record of Dog 2, constructed as described for figure 1. The nitrogen loss of this dog during depletion was considerably less than that of the other two and correspondingly less nitrogen was required to establish equilibrium.

was reversed, and all three dogs retained more nitrogen from the casein hydrolysate than from Mixture V_u. The difference in retention in favor of the casein hydrolysate, was even more striking when the injection level was octupled. The dogs retained 4.8, 3.1 and 2.1 gm of nitrogen daily when the hydrolysate was injected, and 1.0, 1.1 and 0.8 gm with Mixture V_u.

Nitrogen retention with Mixture XII did not equal that of the casein hydrolysate or Mixture V_u. It was injected at only

TABLE 3

Summarized nitrogen balances. Gm per day.

	"KIG"	"PART POLICE"	"POLICE"	AVERAGE FOR 3 DOGS
Casein hydrolysate				
Low level	− 0.11	− 0.19	− 0.27	− 0.19
× 2	+ 0.91	+ 0.31	+ 0.59	+ 0.60
× 4	+ 1.82	+ 1.14	+ 1.49	+ 1.48
× 8	+ 4.78	+ 3.14	+ 2.06	+ 3.33
× 8	+ 3.20	+ 1.29	+ 1.56	+ 2.02
Mixture V_u				
Low level	− 0.04	+ 0.28	+ 0.25	+ 0.16
× 2	+ 0.72	+ 0.87	+ 0.73	+ 0.78
× 4	+ 1.03	+ 1.08	+ 0.79	+ 0.97
× 8	+ 0.81	+ 1.09	+ 1.21	+ 1.04
Mixture XII				
Low level	− 0.75	− 0.22	− 0.06	− 0.34
× 2	− 0.33	+ 0.27	+ 0.06	+ 0.00

two levels since it had to be given much more slowly than the other two preparations in order to avoid nausea and vomiting.

For careful comparison, the summarized nitrogen balance data are given in table 3 for the individual dogs. The 3 dogs behaved similarly to all three supplements, so that the average values are representative. These show that at the two lowest levels of suplementation Mixture V_u was more effective for retention than the casein hydrolysate, but that at the two higher levels the situation was reversed. To determine whether the less efficient retention of Mixture V_u at the

higher levels was due to the sequence of supplementation, or perhaps to the decrease in nitrogen need after so much retention on the casein hydrolysate, a second period using the octupled level of the hydrolysate was given. It is evident from the charts and from table 3 that retention was still high, and was considerably greater than that with the amino acid (V_u) mixture.

Weight. The close parallelism between weight and nitrogen balance is best seen from figure 1. Weight declined during depletion, and was stationary when minimal levels of nitrogen were given, and increased slowly when the nitrogen level reached adequacy.

Plasma albumin. As shown in figure 1, the plasma albumin value rose from 1.6 gm per cent at the end of the depletion period to 2.8 gm per cent at the end of intravenous supplementation. This change does not measure the rapidity of albumin regeneration with parenterally administered nitrogen since the level given during most of the experimental period was inadequate for maximal protein synthesis. The other two dogs (figures 2 and 3) also showed changes in plasma albumin of about the same magnitude.

Hematocrit. The hematocrit values for all three dogs declined during the experimental period. We have no explanation. The experimental diet contained a sufficient amount of iron.

Caloric intake. Except for the caloric value of the nitrogen given by vein the caloric intake was maintained at 50 cal. per kilo body weight. At the octupled level, the supplement increased the intake of Dogs 1 and 3 by about 20 cal. per kg and Dog No. 2 about 10 cal. At the lower nitrogen levels the calories supplied by the supplements did not significantly affect the caloric intake; nor did this increase at the higher level affect the findings.

Excretion. As indicated at the bottom of all three figures the fecal excretion of nitrogen was not modified by the level of intravenous supplementation. The urinary excretion

responded promptly to the injection level, and determined the magnitude of the nitrogen balance.

Excretion of amino acids and peptides. The excretion of amino acids and peptides during all of the periods has been measured. The ninhydrin procedure of Van Slyke et al. ('43) was used to measure the amino nitrogen in the urine. The urine was subsequently hydrolyzed by boiling 10 ml with 10 ml of concentrated hydrochloric acid for 12 hours and the amino nitrogen value re-determined. The difference was attributed to peptides.

Figures 4 and 5 show the magnitude of amino acid excretion as measured by the amino nitrogen. It is evident, when the same levels of supplementation are compared, that the crystalline mixtures resulted in a much greater excretion of amino acids than did the hydrolysate. In all probability this is due to the excretion of unnatural forms of the amino acids, but rapid filtration of amino acids through the kidney glomeruli may contribute to the total value. It is not due to differences in rate of injection. The values recorded above the curves indicate the average number of minutes required daily for the injection of the particular supplement. In figure 4, for example, it required an average of 183 minutes daily to inject the quadrupled level of the hydrolysate, and 277 minutes to inject the same level of crystalline acids (V_u). At both

Fig. 4 Urinary amino nitrogen excretions of Dog 1. The periods at the extreme left and right of the chart indicate the excretion when no infusions were administered. The solid black columns are periods during which the enzymic hydrolysate (Amigen) was injected; the dotted columns are periods during which the ten crystalline essential amino acids and glycine (V_u) were injected, and the ruled columns are periods during which the mixture of ten essential and ten unessential amino acids (XII) was given. The nitrogen injection level is indicated below the columns; thus "4 × V_u" indicates that the V_u mixture was given at 4 times the minimal level. Each individual column represents the average daily 24-hour excretion for periods of 3 or 4 days. Each supplement was given for not less than 10 days. The numbers at the top of the columns are the average minutes required to administer the infusion.

Fig. 5 Urinary amino nitrogen excretions of Dogs 2 and 3. Description of the chart is given under figure 4. The amino nitrogen excreted after the injection of the crystalline mixtures is always greater than following the hydrolysate.

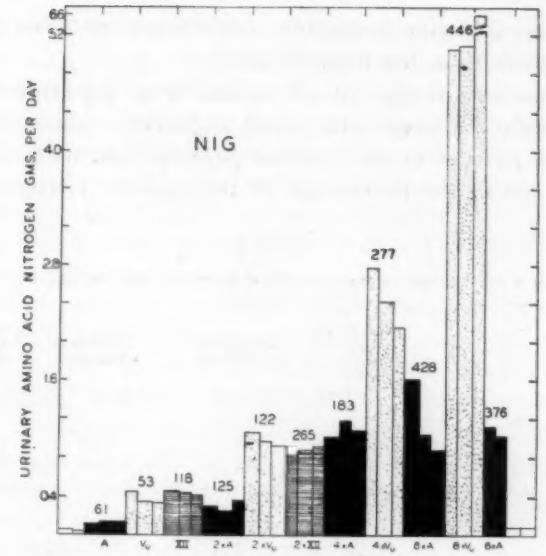

Figure 4

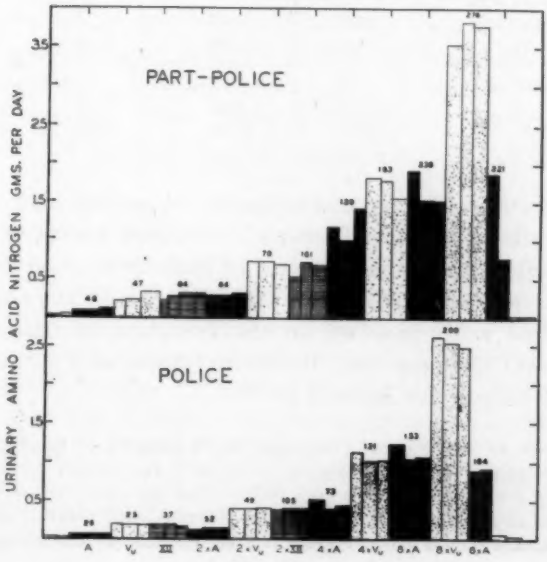

Figure 5

of the high injection levels, the crystalline acids were injected more slowly than the hydrolysate.[5]

The amino nitrogen of all urines from injections of the hydrolysate increased after acid hydrolysis, indicating that a certain amount of the injected peptides had been excreted. Table 4 shows the percentage of the injected nitrogen which

TABLE 4

Per-cent of injected nitrogen excreted as amino and peptide nitrogen.

	INJECTION TIME MINUTES DAILY	AMINO ACID NITROGEN	PEPTIDE NITROGEN	TOTAL NITROGEN IN BOTH FORMS
Minimum level				
Amigen	44	3.55	9.26	12.81
Mixture V_a	42	15.37	1.01	16.38
2 × minimum level				
Amigen	87	6 82	11.22	18.04
Mixture V_a	80	20.40		20.40
4 × minimum level				
Amigen	125	14.09	11.78	25.87
Mixture V_a	187	27.08	0.59	27.67
8 × minimum level				
Amigen	273	10.65	12.08	22.73
Mixture V_a	307	31.18	0.92	32.10

was excreted as amino acid nitrogen, as peptide nitrogen, and in total when the values from all 3 dogs were averaged.

The table shows that the sum of both forms excreted when the different supplements were given is nearly the same. The crystalline acids resulted in the excretion of only slightly more total nitrogen than the hydrolysate, and this was significant only at the highest level.

[5] This rate of injection was determined by the tendency of the dogs to vomit. At the octupled level, for example, the hydrolysate was injected for 50 days and there were 5 days on which the dogs vomited, but the same level of Mixture V_a resulted in an episode of vomiting on 21 of the 30 days on which it was injected. This accounted for the slower average rate of injection for this material.

By comparing the amount of peptide nitrogen injected with the peptide nitrogen excreted, it was determined that 40 to 50% of the injected peptide nitrogen appeared in the urine. This fact needs further study since we have found that pure crystalline dipeptides are utilized when given intravenously. The average chain length of the peptides in the casein hydrolysate approximated 3.4 when the method of calculating the chain length as described by Stein et al. ('44) was used.

DISCUSSION

This comparative study of nitrogen retention when an enzymic hydrolysate and mixtures of amino acids are given intravenously indicates that the essential amino acid composition of a substance will not necessarily determine its value. The administration of 7.65 gm of essential amino acids as the hydrolysate was almost as effective as 16.23 gm of the crystalline essential acids (table 2). At higher levels, but at the same ratio of intake the smaller quantity from the hydrolysate was much more effective in accomplishing nitrogen retention. This difference may be partially explained by the presence of unnatural isomers in the synthetic amino acids, and partially by rapid excretion. But it also indicates to us that the body requires certain amino acids which are not classified as essential. The formation of new protein requires the presence of a number of unessential amino acids and if they are not specifically supplied in the diet, the essential acids will be utilized to form them. It is thus conceivable that if essential amino acids constitute the principal nitrogen of the diet, that more would be required than if certain quantities of unessential amino acids were also given.

The observations further indicate that the hydrolysate can probably be modified by the addition of amino acids to the end that nitrogen retention on minimal levels can be improved. Such studies are now in progress.

Nausea and vomiting accompany the rapid administration of protein hydrolysates and amino acids (Cox and Mueller, '44 a, b; Madden et al., '45 a). Our present observations with

three sources of amino acid nitrogen indicate that the two crystalline mixtures were more prone to cause such disturbances than an enzymic hydrolysate. No satisfactory explanation for the tendency to emesis caused by parenteral administration of amino acids has been found. It is worthy of note that Mixture V_u contained no glutamic (Madden et al., '45 b) or aspartic acid (Madden et al., '45 a) and did contain glycine (Madden et al., '44), and that its tendency to cause vomiting was greater than that of the hydrolysate. The level of amino nitrogen in the blood has been suggested as the factor provoking emesis but this is far from a satisfactory index (Cox and Mueller, '43). Madden's observations indicate that certain mixtures of amino acids provoke less vomiting than others but in our experience dogs vary widely in their reaction to a given mixture on successive days so that it is impossible to incriminate any one essential amino acid (Cox and Mueller, '44 a).

CONCLUSION

An enzymic casein hydrolysate and two mixtures of crystalline amino acids have been compared for their ability to promote nitrogen retention in protein depleted dogs when given intravenously at varying levels of nitrogen intake.

At levels just sufficient to establish nitrogen equilibrium the mixture of ten essential amino acids and glycine (V_u) was slightly more effective in promoting nitrogen retention than the other two substances. At higher levels of intake the hydrolysate was considerably more effective than the V_u mixture.

Urinary excretion of amino acid nitrogen was always greater with the crystalline mixtures than with the hydrolysate. Conversely, more peptide nitrogen appeared in the urine when the hydrolysate was given. The sums of nitrogen lost in both forms were approximately equal although the crystalline mixture was given more slowly than the hydrolysate in order to avoid emesis.

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THE INFLUENCE OF SOURCES OF PHOSPHORUS ON THE RELATIVE EFFICIENCY OF VITAMIN D_3 AND COD LIVER OIL IN PROMOTING CALCIFICATION IN POULTS[1]

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ONE FIGURE

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The work of Fritz, Hooper and Moore ('45), which has been essentially confirmed by Evans and Brant ('45), would indicate that the discrepancies reported in the literature pertaining to the vitamin D requirements of poults, can be explained in part by the wide variation in the levels of either calcium or phosphorus that have been used in the test diets. Previously Bird ('44) and Boucher ('44) had indicated that this might be a possible explanation.

That vitamin D_3 is approximately two times as efficient as fish liver oil in promoting calcification in the poult, chick unit for chick unit, has been reported by Boucher ('44) and Bird ('44). Fritz et al. ('45) observed the same trend but quantitatively the differences noted by them were much less marked than those reported by either Boucher or Bird. The "Borden Diet" employed by Fritz and co-workers analyzed 1.51% calcium and 1.0% phosphorus. The phosphorus content of the "Borden Diet" was greater than that of the diets used by either Bird or Boucher and in addition must have contained

[1] Supported in part by a grant from the Yantic Grain and Products Company, Norwich, Connecticut.

a greater amount of readily available phopshorus. This might explain why extreme differences in response were not observed by Fritz and his co-workers. It should be noted that the diet employed by Boucher contained a relatively small amount of non-phytin phosphorus. We have estimated that his ration contained less than 0.2% of non-phytin phosphorus. The composition of Bird's diet, while similar to that of Boucher's but containing a greater per cent of phosphorus (0.82%), may also have been low in readily available phosphorus (vide infra).

It is conceivable that the results obtained by Boucher and others and interpreted as a species specificity, could be explained on the basis of the availability of the phosphorus as well as the absolute level of this mineral in the diets. Moreover, the wide differences of opinion in regard to the vitamin D requirements of the poult, may, in part at least, have originated because of differences in readily available phosphorus in the rations. In Boucher's work as well as that of Bird, the vitamin D carriers were first assayed on the A.O.A.C. diet with chicks, which is relatively high in inorganic phosphorus. The vitamin supplements were then incorporated in turkey rations relatively low in inorganic phosphorus considering the greater known mineral requirements of the poult. It should be emphasized that poults were not fed the A.O.A.C. diet nor were chicks fed the poult diets. However, from pilot experiments in progress at this station, it should be stated that there is some indication that the A.O.A.C. chick diet when fed to poults may not contain a sufficient amount of calcium and of readily available phosphorus to assay adequately vitamin D carriers.

It occurred to one of us (H.M.S.) that the source of vitamin D might account for differences observed in bone ash of chicks on a diet high in phytin phosphorus. This idea was developed by Singsen and Mitchell ('45) who have shown that activated animal sterols (Delsterol) are 1.34 times as effective as a pure cod liver oil in promoting calcification in the chick when the chick must rely upon phytin phosphorus to meet its require-

ments. That poults subjected to a similar experimental design also exhibit this differential in response is indicated in the data presented here.

The objectives of this work were: (1) to determine the response of turkey poults to vitamin D from activated 7-dehydrocholesterol and a pure cod liver oil when incorporated in a simplified diet in which all of the phosphorus was readily available and (2) to measure the effectiveness of these two sources of vitamin D in promoting the utilization of the phosphorus of phytin when the poult is forced to rely upon phytin to meet its phosphorus requirements.

EXPERIMENTAL

The low phosphorus basal mixture consisted of white table corn meal [2] 67.3, casein 12.0, gelatin 3.0, primary grown yeast 4.0, bagasse 4.0, liver meal 3.0, wheat germ oil 1.0, $CaCO_3$ 2.23, KCl 0.5, and salt mixture 1.6.[3] The vitamin supplement per 100 pounds of the ration consisted of calcium pantothenate 635 mg, pyridoxine 227 mg, riboflavin 227 mg, vitamin K 18 mg, thiamine chloride 95 mg, nicotinic acid 95 mg, inositol 227 mg, para amino benzoic acid 227 mg, choline chloride 45.4 gm and vitamin A 800,000 I.U. On analysis this basal mixture was shown to contain 1.21% calcium and 0.23% phosphorus.

Two experiments were conducted in which the vitamin D from a pure cod liver oil was compared with activated 7-dehydrocholesterol, the principle dietary variation being the source of phosphorus used.

In experiment 1, $NaH_2PO_4 \cdot H_2O$ (1.37%) was added to the basal diet to bring the total phosphorus level to 0.61%, all of which was readily available. In experiment 2, calcium-magnesium phytate (2.09%) raised the total phosphorus level of the basal diet to 0.55%. Phytin phosphorus by analysis,

[2] A de-germed, de-braned product especially prepared for table use and containing only 0.07% phosphorus of which less than 0.01% is phytin.

[3] 1000 gm NaCl, 10 gm KI, 150 gm $MnSO_4 \cdot H_2O$, 50 gm $FeSO_4 \times H_2O$, 136 gm $MgSO_4$, 5 gm $ZNSO_4$, 5 gm $CaSO_4 \cdot 5\ H_2O$, 200 gm $NaSiO^3$. $6\ H_2O$, 50 gm $Al_2(SO_4)_3$, 2 gm $Co(CH_3COO)_2 \cdot 4\ H_2O$.

accounted for 55.4% of the total phosphorus in the diet. It was the original intent to have the level of phosphorus identical in both rations.

The pure cod liver oil and the activated 7-dehydrocholesterol were assayed in our laboratory by the A.O.A.C. procedure and incorporated in the diets on a chick unitage basis.[4] Twenty bronze day-old poults, all originating from the same breeding stock maintained by the station were placed in each experimental lot. They were confined to electric brooding compartments located in a room where all daylight was excluded. At the conclusion of the experimental period all surviving poults were weighed and then sacrificed for the removal of the left tibiae. The bones were cleaned, extracted in alcohol for 48 hours, ether for 24 hours and then ashed individually. Because of the extreme degree of rachitis manifested by all lots fed the phytin phosphorus diet this experiment was terminated at 21 days. The results are presented in table 1.

DISCUSSION

An examination of the data presented in table 1 (Expt. 1) shows clearly that 7-dehydrocholesterol when incorporated in the inorganic phosphorus diet was no more efficient in promoting calcification than was cod liver oil when the two sources of vitamin D were fed on an equal chick unitage basis. At the 80 unit level the 7-dehydrocholesterol did give a higher percentage of bone ash than did the cod liver oil. However, this is thought to be due to chance since all other levels in both series are essentially equal.

Where the design of the experiment is such that the poult is forced to rely upon phytin phosphorus for bone formation (Expt. 2), the greater efficiency of vitamin D_3

[4] The 7-dehydrocholesterol in corn oil had a manufacturer's assay of 200,000 A.O.A.C. chick units per gm. One gm of this concentrate was diluted with 799 gm of corn oil. This gave sufficient material to carry out both the chick assays and the turkey experiments without making new dilutions. The diluted 7-dehydrocholesterol assayed 250 A.O.A.C. units per gm. The cod liver oil which carried a manufacturer's potency of 250 A.O.A.C. units/gm was assayed by us to contain 255 A.O.A.C. units/gm.

manifests itself at each level of vitamin D intake. In poult nutrition, as in the nutrition of the chick, it would appear that the greater effectiveness of the 7-dehydrocholesterol is demonstrated most markedly when the ration is high in phytin phosphorus and low in inorganic phosphorus.

The efficacy ratios submitted in the last column of table 1 were estimated by fitting a straight line equation, $Y = 22.173 + 0.013\ X$ to the data points of the cod liver oil groups represented in the phytin phosphorus series. The percentage ash values for each level of 7-dehydrocholesterol fed were

TABLE 1

	VIT. D UNITS/ 100 GM DIET	PURE COD LIVER OIL		ACTIVATED 7-DEHYDROCHOLESTEROL		EFFICACY RATIO
		Av. wt.	% boneash	Av. wt.	% boneash	
Summary	0	117.4 (5)[1]	22.45	117.4 (5)	22.45	
expt. 1 with	40	204.3 (17)	33.03	199.5 (14)	33.02	
$NaH_2PO_4 \cdot H_2O$	80	219.3 (12)	40.13	286.4 (19)	43.24	
28 days	160	248.6 (14)	45.29	277.5 (13)	46.52	
	200	275.5 (17)	46.21	295.1 (16)	46.06	
Summary	0	112.4 (20)	23.26	112.4 (20)	23.26	
expt. 2 with	40	127.3 (16)	23.37	128.1 (16)	25.91	6.80
phytin	80	117.5 (15)	24.12	130.8 (19)	27.18	4.65
phosphorus	160	125.2 (14)	24.82	141.3 (17)	27.43	2.40
21 days	200	132.7 (17)	24.61	139.6 (17)	27.70	2.04
	320	140.6 (12)	27.51	148.3 (19)	28.92	1.55
	640	133.3 (18)	27.88	157.3 (19)	31.06	1.04

[1] Figures in parentheses = no. of surviving poults.

plotted on the graph (fig. 1). For any given value, a line is run parallel to the abscissa to a point where it cuts the line of best fit. At this point a line is dropped perpendicular to the abscissa where the corresponding units of vitamin D from cod liver oil necessary to produce an equivalent bone ash may be read. This figure divided by the number of A.O.A.C. chick units of 7-dehydrocholesterol actually fed gives the efficacy ratio.

There are two points of interest to be noted in experiment two. (1) Although the highest level of vitamin D fed in experiment 2 was twice that of the highest level in experiment 1, the

bone ash for this high level (Expt. 2) is less than that for the lowest level of vitamin D in experiment 1. This indicates definitely that calcium magnesium phytate is not a readily available source of phosphorus. One should not, however, infer that the phosphorus of natural plant phytin is necessarily as unavailable (vide infra). (2) As the vitamin D level in the diet increases, bone ash likewise increases. However, the increase in per cent bone ash at the higher levels, per unit

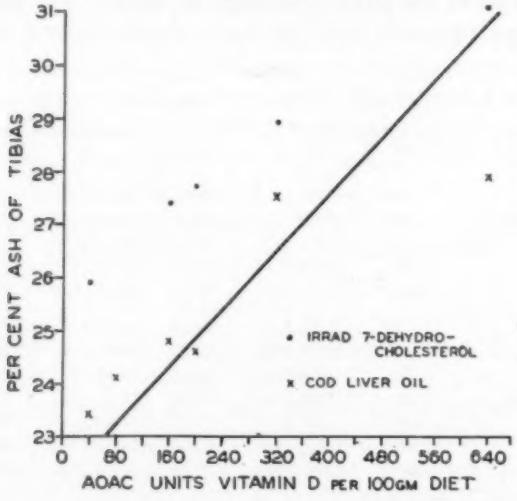

FIG 1 EFFICACY OF 7-DEHYDROCHOLESTEROL AS COMPARED WITH COD LIVER OIL FOR PROMOTING BONE ASH IN POULTS

of vitamin D fed, is considerably less than at the lower levels. Thus we have the greater efficacy ratios for 7-dehydrocholesterol appearing at the lower levels.

Is it possible to correlate these findings with the discrepancies that exist in the literature on the requirements of the turkey poult for vitamin D?

Common ('40) has analyzed some 30 constituents of English poultry feeds for phytin and concludes that in the usual mixed feed some 75% of the phosphorus derived from the cereals

will be in the form of phytic acid. One of us (E.P.S.) has analyzed corn, oats, bran and middlings for phytin with a slightly modified procedure from that used by Common. These analyses seem to run, on the average, some 5% higher than those of Common. This may be due to either a difference in the technique involved or the actual content of phytic acid in the sample analyzed. Common's findings of a considerable spread in the phytic acid content of different samples of feedstuffs has been confirmed in this laboratory (i.e., different samples of wheat may analyze from 52–72% of the total phosphorus as phosphorus from phytin). That phytin phosphorus from different sources may also vary in its effectiveness in fulfilling the chicks requirements for this mineral is also indicated by some unpublished data of one of us (E.P.S.).

In view of the variations noted above, it is difficult to calculate the absolute values for phytin phosphorus in the various diets used by investigators in studying this problem. However, in the case of Boucher's diet (0.63% total phosphorus) which contained no added mineral phosphorus supplement, there would seem to be little question that the differences noted can be explained on the basis that the poults were forced to rely on phosphorus from phytin to satisfy their requirements. Bird's diet, on the other hand, contained 1.5% defluorinated superphosphate which, if it were all available, should furnish 0.399% inorganic phosphorus. On the basis of the data presented in table 1, this amount of inorganic phosphorus plus the 0.421% phosphorus (0.201% non-cereal phosphorus) from organic sources in the diet should have resulted in more equal calcification for all sources of vitamin D used. However, Matterson et al. ('45) and Bird et al. ('45) have shown that in the case of the chick and Ellis et al. ('45) in the case of rats, defluorinated superphosphate is not as effective for promoting bone calcification as is tri calcium phosphate or bone meal and if this same condition holds for the poult it would seem reasonable to suppose that the differences noted by Bird can be traced to the differences in

effectiveness of various sources of vitamin D in promoting the utilization of phytin phosphorus in his diet. The diet of Carver and Rhian ('42) was so high in non-cereal phosphorus (0.60%) that one would expect, on the basis of data presented here, to find but slight, if any, difference in the effectiveness of fortified oils as compared with cod liver oil, as indeed they did not. Hammond's ('41) diet (0.48% non-cereal phosphorus) would seem to be approaching the normal requirement of the poult for this type of phosphorus, since his data show a requirement of 80 A.O.A.C. chick units from reference cod liver oil. It should be noted, however, that even at this level of non-cereal phosphorus, activated animal sterols exhibit a greater efficiency, 50 units of activated animal sterol being equivalent to 80 units of reference cod liver oil.

The one discrepancy that stands out in sharp contrast to the hypothesis developed above is the work of Jukes and Sanford ('39). Their diet is calculated to contain 1.0% of total phosphorus and 0.638% of non-cereal phosphorus. This is a greater percentage of non-cereal phosphorus than appears in any of the other diets discussed above and if experimental conditions were comparable should have resulted in a much greater bone ash than these workers report. It seems futile to speculate as to the cause of this disagreement. Nevertheless, it is interesting to note that their later paper (Sanford and Jukes, '44), seems to give us a clue that some factor was operating to depress bone calcification. In their original work, U.S.P. cod liver oil No. 1 when fed at the 80 unit level gave a bone ash of 36.64%. In their later work, with a diet of the same constituent and percentage composition and using U.S.P. Reference Oil No. 2, they report a bone ash figure of 43.4% at the 80 unit level.

One may also note that with their diet No. 2, which more nearly approaches the other diets in mineral content and yet should furnish a sufficient quantity of total phosphorus for bone calcification, they report a lower bone ash at the 80 unit level with Reference Oil No. 2 than they do for diet No. 1 with the same reference oil (40.4% as compared to 43.4%).

CONCLUSIONS

1. When the diet contains adequate amounts of readily available phosphorus, vitamin D from activated 7-dehydrocholesterol and from cod liver oil produce approximately equal calcification in poults.

2. Vitamin D from activated 7-dehydrocholesterol is more effective than that from cod liver oil in promoting calcification in poults which are forced to rely upon phytin phosphorus to meet their requirements for this mineral. This difference in efficacy ranges from 6.8:1 to 1.04:1 and varies inversely with the level of vitamin D in the feed.

3. With but one exception (Jukes and Sanford, '39) the discrepancies which appear in the literature as to the vitamin D requirement of turkey poults can be explained for the most part at least, on the basis of the availability of the phosphorus used with the various sources of vitamin D in the diet. When the diet is relatively high in the percentage of non-cereal phosphorus, both forms of vitamin D are effective at the lower levels reported. When the diet is relatively high in cereal phosphorus the vitamin D from cod liver oil is less effective and the greater efficacy of activated animal sterols manifests itself.

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SOME INTERRELATIONSHIPS OF DIETARY IRON, COPPER AND COBALT IN METABOLISM[1]

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The curative effect of cobalt in coast disease in South Australia, enzootic marasmus of Western Australia, bush sickness of New Zealand, nakuriutus of Kenya, pining disease of Scotland, wasting disease of Western Canada, salt sickness of Florida, lake shore disease of Michigan and Wisconsin and Burton-ail of New Hampshire and Massachusetts (Marston, Thomas, Murname, Lines, McDonald, Moore and Bull, '38; Bowstead, Sackville and Sinclair, '42; Killham, '41; and Keener, Percival and Morrow, '44) has led to the hypothesis that cobalt is an essential element for certain animals. In order to determine the rôle played by cobalt in normal metabolism and its relationship, if any, to iron and copper, this project was undertaken. A microchemical method developed for cobalt analyses on high ash samples made this work possible.

The initial purpose was to estimate cobalt retention at levels in the food characteristic of our diet; to determine the effect of iron and copper on cobalt, and of cobalt on iron and copper retention; and to investigate the disputed interrelationships of iron and copper (Schultz, '40), as well as the validity of application of data on iron and copper anemic animals to normal animals.

[1] The material in this paper was presented before the meetings of the Biological Chemistry section of the American Chemical Society at New York, September 14, 1944.

The plan of the experimentation was to vary systematically the iron, copper and cobalt content of an otherwise good diet and determine the response in food intake, growth, hemoglobin level and metal storage and retention in the healthy albino rat becoming anemic.

EXPERIMENTAL

Test diets

A basal diet low in iron, copper and cobalt but adequate for fair growth and hemoglobin level was prepared. Because of reports by Ahmad and McCollum ('39), Morris ('40) and Sylvester and Lampitt ('40) of the low cobalt content of milk and milk powder, recently verified by Ellis,[2] the latter was selected as the main constituent of the basal diet.

The composition of the basal diet prepared was as follows: Whole cow's milk powder, 48.5%; washed sucrose,[3] 48.5%; washed Celluration,[4] 2.0%; C.P. sodium chloride, 1.0%; and thiamine hydrochloride, 0.00034%. The washed sucrose was prepared by slowly extracting super-fine sucrose in a long glass Tswett column with 95% ethanol, to which a trace of ammonia had been added, then with ether and finally drying on exposure of a thin layer, protected from dust, to the air. The washed Celluration was prepared by soaking Celluration in approximately 0.2 molar hydrochloric acid solution for 24 hours, with occasional shaking. The solution was filtered off and the extraction repeated the following day. The Celluration then was rinsed several times with redistilled water and dried at 100°C. in Pyrex dishes. The basal metal-low diet was calculated to contain 13% protein, 0.46% calcium, Ca/P = 1.3, 0.0005% thiamine, 0.0008% riboflavin, 8.0 I.U. vitamin A and 4.4 Cal. per gm diet.

[2] Private communications with G. H. Ellis, U. S. Plant, Soil and Nutrition Laboratory, Ithaca, N. Y.; also published in part in Ind. Eng. Chem., Anal. Ed., vol. 17, p. 254 (1945).

[3] Super-fine sucrose, National Sugar Refining Company, New York.

[4] Celluration (70% alpha cellulose, approximately 29% simple and hydrocelluloses and a total ash less than 1.0%), Fischer Scientific Company, N. Y.

This basal diet was analyzed for its content of iron, copper and cobalt and five diets of varying iron, copper and cobalt content devised for the metal studies as shown in table 1. For comparison, the calculated metal content of diet 13 of this laboratory also is given in this table.

Animal technique

Young healthy albino rats of a strain which had been for several generations on adequate diets of whole wheat, whole milk and table salt (diet 13, diet 130 and diet 140 [5]), were selected from our stock colony. They were weaned at 28 days of age and killed or placed on the experimental diets at 31 to

TABLE 1

Metal composition of diets fed animals.

DIET NO.	METAL CONTENT OF DIETS		
	Iron	Copper	Cobalt
	p.p.m.	*p.p.m.*	*p.p.m.*
I (Basal; low Fe + Cu + Co)	2.4	0.67	0.003
II (Basal + Fe + Cu)	4.2	5.5	0.003
III (Basal + Fe + Co)	4.2	0.67	0.083
IV (Basal + Fe + Cu + Co)	3.4	5.5	0.083
V (Basal + Fe + Cu + Co)	4.2	5.5	0.083
13 [1] (Sherman stock diet)	31	6.2	0.08

[1] Calculated cobalt content of diet based on data of Ahmad and McCollum ('39).

33 days old. The animals were divided into six groups so as to equalize, as experimentally practicable, their weight, sex, hemoglobin level and heredity. Animals to be subjected to the experimental diets were placed in individual galvanized wire cages which had been coated recently with aluminum paint. The cages had raised screen bottoms.

Food and distilled water were available to all animals ad libitum. Food records were kept. All animals were weighed weekly and their hemoglobin level determined semimonthly.

[5] 31.6 to 32.9% whole cow's milk powder, 63.3 to 65.8% whole wheat and 5.1 to 1.3% sodium chloride.

At 0, 30 and 60 experimental days, an equal number of animals from each of the five diets was killed and the total carcass, minus the gastrointestinal tract, analyzed for iron, copper and cobalt.

Analytical methods

Hemoglobin measurements were made by a modified Hill and Pincock ('41) method, using 0.04% ammonia solution. Spectrophotometric measurements on hemoglobin solutions were made at 540 mμ.

Diets and rat carcasses were analyzed, without grinding, by a modified Marston and Dewey ('40) method. A sample was wet and dry ashed repeatedly at low temperatures for short intervals of time. The organic-free ash remaining was refluxed with 8 N hydrochloric acid and the iron present extracted with isopropyl ether. Final determination of iron was made with o-phenanthroline. After evaporation almost to dryness of the ether extracted aqueous layer, containing the copper and cobalt, the residue was dissolved in water and the solution formed adjusted to pH 2.5–2.8. The copper was then extracted with dithizone in chloroform and finally determined with sodium diethyldithiocarbamate; at this point, cobalt was extracted with α-nitroso-β-naphthol in carbon tetrachloride. Its final determinization was made with nitroso-R-salt.

RESULTS AND DISCUSSION

The effect of varying ratios of iron, copper and cobalt in five different diets is shown on food intake and growth (table 2), hemoglobin level (table 3), metal content of fresh carcass after 30 days and 60 days on the experimental diets (table 4) and metal retention (table 5).

Food intake, growth and hemoglobin level decreased almost as rapidly in animals on low copper diet III as in animals on low iron, copper and cobalt diet I. Doubling the iron and increasing the cobalt thirtyfold in the basal diet, in the absence of copper, did not benefit the animal markedly in these respects. The rapid drop of hemoglobin level of animals on

TABLE 2

Growth and food intake of animals on diets of varying metal content.

DIET	NO. OF RATS Period (days) 0–30	30–60	SEX	AVERAGE BODY WEIGHT PER RAT Days rat on diet 0	14	30	42	60	AVERAGE DAILY FOOD INTAKE PER RAT Period (days) rat on diet 0–14	14–30	30–42	42–60
				gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
I	4	2	M	53 ± 3[1]	79 ± 5	108 ± 9	133 ± 6	141 ± 12	83 ± 3	110 ± 6	95 ± 5	127 ± 8
	4	2	F	52 ± 2	81 ± 5	105 ± 5	123 ± 3	124 ± 11	89 ± 6	112 ± 5	81 ± 10	111 ± 12
	8	4	M + F	53 ± 3	80 ± 5	107 ± 7	127 ± 5	131 ± 12	86 ± 5	111 ± 6	88 ± 8	119 ± 10
II	4	2	M	52 ± 3	82 ± 4	119 ± 5	153 ± 3	177 ± 6	83 ± 9	126 ± 6	116 ± 2	178 ± 3
	4	2	F	50 ± 5	80 ± 2	116 ± 5	139 ± 14	159 ± 19	91 ± 4	131 ± 7	108 ± 11	175 ± 10
	8	4	M + F	51 ± 4	81 ± 3	118 ± 5	146 ± 9	168 ± 13	87 ± 7	129 ± 7	112 ± 7	177 ± 7
III	4	2	M	52 ± 4	77 ± 4	101 ± 6	118 ± 14	126 ± 19	82 ± 5	105 ± 11	94 ± 10	122 ± 14
	4	2	F	49 ± 5	73 ± 7	95 ± 11	106 ± 14	102 ± 7	81 ± 10	104 ± 14	77 ± 7	103 ± 0
	8	4	M + F	51 ± 5	75 ± 6	98 ± 9	112 ± 14	114 ± 13	82 ± 8	105 ± 13	86 ± 9	113 ± 7
IV	4	2	M	55 ± 3	81 ± 8	117 ± 10	149 ± 4	177 ± 3	85 ± 9	126 ± 10	113 ± 3	179 ± 7
	4	2	F	49 ± 5	80 ± 6	114 ± 8	142 ± 8	160 ± 11	87 ± 7	124 ± 8	107 ± 4	170 ± 1
	8	4	M + F	52 ± 4	81 ± 7	116 ± 9	146 ± 6	169 ± 7	86 ± 8	125 ± 9	110 ± 4	175 ± 4
V	4	2	M	50 ± 4	78 ± 4	112 ± 6	137 ± 5	159 ± 6	88 ± 4	125 ± 4	102 ± 2	165 ± 4
	4	2	F	51 ± 5	80 ± 10	110 ± 8	127 ± 10	142 ± 13	91 ± 7	128 ± 5	99 ± 5	157 ± 11
	8	4	M + F	51 ± 5	79 ± 7	111 ± 7	132 ± 8	151 ± 10	90 ± 6	127 ± 5	101 ± 4	161 ± 8

[1] Average deviation of a single observation from the arithmetical mean.

TABLE 3

Hemoglobin level of animals on diets of varying metal content.

DIET	NO OF RATS		SEX	AVERAGE HEMOGLOBIN LEVEL PER RAT				
	Period (days)			Days rat on diet				
	0–30	30–60		0	14	30	42	60
				gm/100 ml	*gm/100 ml*	*gm/100 ml*	*gm/100 ml*	*gm/100 ml*
I	4	2	M	12.65 ± 0.48 [1]	9.68 ± 0.52	5.66 ± 0.63	3.49 ± 0.05	2.47 ± 0.14
	4	2	F	12.54 ± 0.48	10.35 ± 0.77	5.73 ± 0.84	4.22 ± 0.45	2.76 ± 0.35
	8	4	M + F	12.60 ± 0.48	10.02 ± 0.65	5.70 ± 0.74	3.86 ± 0.25	2.62 ± 0.25
II	4	2	M	12.35 ± 0.68	10.56 ± 0.73	8.59 ± 0.62	8.16 ± 0.04	9.26 ± 0.44
	4	2	F	12.62 ± 0.15	10.94 ± 0.28	9.32 ± 0.81	9.22 ± 0.44	10.59 ± 0.63
	8	4	M + F	12.49 ± 0.42	10.75 ± 0.51	8.96 ± 0.72	8.69 ± 0.24	9.93 ± 0.54
III	4	2	M	12.44 ± 0.85	9.96 ± 1.02	6.19 ± 1.58	5.70 ± 0.05	3.30 ± 0.25
	4	2	F	13.23 ± 0.57	10.52 ± 0.44	6.87 ± 0.92	5.88 ± 0.27	2.96 ± 0.15
	8	4	M + F	12.84 ± 0.71	10.24 ± 0.73	6.53 ± 1.25	5.79 ± 0.16	3.13 ± 0.20
IV	4	2	M	12.06 ± 0.52	11.52 ± 0.26	9.02 ± 0.32	7.88 ± 0.16	7.96 ± 0.33
	4	2	F	12.28 ± 0.46	9.62 ± 0.44	7.84 ± 1.20	7.17 ± 1.08	8.11 ± 0.53
	8	4	M + F	12.17 ± 0.49	10.57 ± 0.35	8.43 ± 0.76	7.53 ± 0.62	8.08 ± 0.43
V	4	2	M	12.24 ± 0.24	11.29 ± 0.38	9.14 ± 0.73	8.79 ± 1.20	9.42 ± 0.83
	4	2	F	12.92 ± 0.51	10.96 ± 1.12	9.54 ± 0.38	9.98 ± 0.74	10.68 ± 0.63
	8	4	M + F	12.58 ± 0.38	11.13 ± 0.75	9.34 ± 0.56	9.39 ± 0.97	10.05 ± 0.73

[1] Average deviation of a single observation from the arithmetical mean.

TABLE 4

Iron, copper and cobalt content of animals on diets of varying metal content.

DIET	DAYS RATS ON DIET	NO. OF RATS	SEX	METAL CONTENT PER RAT			METAL CONTENT PER 100 GM FRESH RAT CARCASS		
				Iron	Copper	Cobalt	Iron	Copper	Cobalt
				mg	μg	μg	mg	μg	μg
—	0	3	M	1.63 ± 0.18 [1]	65.8 ± 4.8	0.06 ± 0.03	3.48 ± 0.16	141.0 ± 0.0	0.11 ± 0.06
		3	F	1.71 ± 0.10	65.5 ± 6.0	0.10 ± 0.06	3.61 ± 0.03	137.0 ± 4.0	0.22 ± 0.16
		6	M + F	1.67 ± 0.14	65.7 ± 5.4	0.08 ± 0.05	3.55 ± 0.10	139.0 ± 2.0	0.17 ± 0.11
I	30	1	M	1.63	63.9	0.55	2.02	79.2	0.68
		2	F	1.63 ± 0.13	49.7 ± 5.6	0.52 ± 0.12	1.92 ± 0.17	58.5 ± 7.2	0.61 ± 0.13
		3	M + F	1.63	56.8	0.54	1.97	68.9	0.65
II	30	1	M	2.66	114.9	0.53	2.61	113.	0.52
		2	F	2.03 ± 0.08	75.4 ± 5.6	0.77 ± 0.21	2.08 ± 0.10	77.1 ± 5.2	0.79 ± 0.21
		3	M + F	2.35	95.2	0.65	2.35	95.1	0.66
III	30	1	M	2.01	58.4	1.16	2.28	66.2	1.31
		2	F	1.66 ± 0.31	51.7 ± 2.7	1.28 ± 0.05	2.09 ± 0.24	66.2 ± 8.5	1.64 ± 0.19
		3	M + F	1.84	55.1	1.22	2.19	66.2	1.48
IV	30	1	M	2.38	108.2	1.45	2.23	102.	1.36
		2	F	1.91 ± 0.09	86.5 ± 3.5	1.44 ± 0.41	2.07 ± 0.05	93.8 ± 6.2	1.57 ± 0.48
		3	M + F	2.15	97.4	1.45	2.15	97.9	1.47
V	30	1	M	3.1	160.	2.3	2.9	150.	2.2
		2	F	2.36 ± 0.53	100.2 ± 6.0	1.53 ± 0.24	2.41 ± 0.35	104.7 ± 14.9	1.57 ± 0.12
		3	M + F	2.73	130.1	1.92	2.66	127.	1.89
I	60	2	M	1.89 ± 0.14	50.1 ± 3.6	0.33 ± 0.03	1.52 ± 0.03	40.7 ± 5.3	0.26 ± 0.01
		2	F	2.13 ± 0.14	46.5 ± 0.1	0.56 ± 0.08	1.94 ± 0.02	42.5 ± 3.2	0.52 ± 0.11
		4	M + F	2.01 ± 0.14	48.3 ± 1.9	0.45 ± 0.06	1.73 ± 0.03	41.6 ± 4.3	0.39 ± 0.06
II	60	2	M	3.21 ± 0.03	143.4 ± 1.3	0.64 ± 0.12	2.01 ± 0.08	89.8 ± 3.4	0.40 ± 0.06
		2	F	3.23 ± 0.20	127.0 ± 1.2	0.77 ± 0.21	2.27 ± 0.13	89.7 ± 11.3	0.57 ± 0.22
		4	M + F	3.22 ± 0.12	135.2 ± 1.3	0.71 ± 0.17	2.14 ± 0.11	89.8 ± 7.4	0.49 ± 0.14
III	60	2	M	2.10 ± 0.32	54.5 ± 6 5	1.17 ± 0 41	1.92 ± 0.02	49.9 ± 2.1	1.03 ± 0.21
		2	F	2.28 ± 0.27	44.8 ± 1.3	1.11 ± 0 10	2.45 ± 0.08	50 0 ± 5.9	1.24 ± 0.22
		4	M + F	2.17 ± 0.30	49.7 ± 3.9	1.14 ± 0.26	2.19 ± 0.05	50.0 ± 4.0	1.14 ± 0.22
IV	60	2	M	2.87 ± 0.01	142.1 ± 7.6	1.80 ± 0 70	1.80 ± 0.01	88.9 ± 4.5	1.12 ± 0.44
		2	F	2.84 ± 0.17	145.7 ± 30.2	2.26 ± 0.24	2.00 ± 0.02	102.0 ± 16 2	1.61 ± 0.25
		4	M + F	2.86 ± 0.09	143.9 ± 18.9	2.03 ± 0.47	1.90 ± 0.02	95.5 ± 10.4	1.37 ± 0.35
V	60	2	M	3.05 ± 0.05	114.4 ± 5 6	1.60 ± 0 70	2.07 ± 0.17	77 6 ± 8 9	1.10 ± 0.5
		2	F	2.90 ± 0.00	126.3 ± 18.0	1.30 ± 0.20	2.32 ± 0.21	102.4 ± 23.6	1.10 ± 0.3
		4	M + F	2.98 ± 0.03	120.4 ± 11.8	1.50 ± 0.50	2 20 ± 0.19	90.0 ± 16.3	1.10 ± 0.4

[1] Average deviation of a single observation from the arithmetical mean.

diet III at the end of 15 days showed inadequate copper storage in the weaned animal. The increase of iron and cobalt of diet III over diet I, while reflected in increased iron and cobalt carcass values, did not change markedly the copper content per 100 gm fresh carcass or copper retention. Cunningham ('31) had found similar results for added inorganic iron alone.

The addition of copper (diet V) to a diet low in copper (diet III) resulted in the expected good biological response. While this increase in copper only slightly increased the iron and cobalt content per 100 gm fresh carcass and cobalt retention, it doubled the iron retention. This observation, on animals becoming anemic, confirms the findings of Bing, Saurwein and Myers ('34), who had obtained results seemingly contradictory to those of Josephs ('32) on anemic animals.

Animals on low cobalt diet II showed a food intake, growth and condition comparable to animals on high cobalt diet V. The addition of cobalt to the diet did not affect the iron and copper content per 100 gm carcass or iron and copper retention. Likewise, the addition of iron and copper to diet I to give diet II did not significantly increase the cobalt content per 100 gm carcass or cobalt retention.

TABLE 5

Calculated retention of dietary iron, copper and cobalt by animals.

METAL	DAYS ON DIET	NO. OF RATS	AVERAGE RETENTION PER RAT, DIETS				
			I	II	III	IV	V
			%	%	%	%	%
Iron	30	3	− 12.0 ± 13[1]	60.0 ± 20	8.0 ± 24	54.0 ± 19	97.0 ± 32
	60	4	35.0 ± 6	73.0 ± 2	31.0 ± 8	73.0 ± 5	66.0 ± 3
Copper	30	3	− 8.3 ± 2.9	1.9 ± 0.9	− 9.8 ± 1.7	2.5 ± 0.5	4.4 ± 1.3
	60	4	− 6.4 ± 0.8	2.5 ± 0.2	− 6.2 ± 1.2	3.0 ± 0.3	2.1 ± 0.3
Cobalt	30	3	75.0 ± 10	90.0 ± 15	7.7 ± 0.6	7.9 ± 0.9	8.9 ± 1.0
	60	4	30.0 ± 5	42.0 ± 6	3.3 ± 0.4	4.9 ± 0.5	3.6 ± 0.6

[1] Average deviation of a single observation from the arithmetical mean.

Food intake and growth were as good for animals on diet IV as on diet V. The slightly lower iron content of diet IV in comparison to diet V was reflected in a lower iron carcass value as well as a lower hemoglobin level in the animal. This small decrease in iron of the diet resulted in no significant change in copper or cobalt content per 100 gm fresh carcass or copper and cobalt retention.

All animals on the five diets increased in total iron and cobalt. However, animals on copper-low diets I and III utilized the copper so poorly that they lost more body copper than they retained from the diets. Animals on the high copper diets II, IV and V stored copper. Likewise, animals on diets I and III had an average copper retention of —6.2 to —6.4%, while those on diets II, IV and V had a 2.1 to 3.0% copper retention. While iron may be rather tenaciously retained by the body, this is not the case for copper which can be lost from body stores in the healthy rat. Although anemic animals generally are considered to retain more metal than normal animals on the same diet, a weaned animal becoming anemic on a copper-low diet actually retained less copper than a litter mate on a good copper diet.

Animals on low copper diets I and III retained only 31 to 35% iron, while animals on high copper diets II, IV and V retained 66 to 73% iron. It is evident that the relatively poor utilization of the iron in the milk diets was due to its low content of copper.

In the case of cobalt, cobalt retention on low cobalt diets (0.003 part per million cobalt) was found to be 30 to 42%, while retention on high cobalt diets (0.08 p.p.m. cobalt) was 3.3 to 4.9%. No retention data for low cobalt diets have yet appeared in the literature. However, Copp and Greenberg ('41) reported that a 12-week-old rat retained, after 96 hours, 2.7% of a single 10 μg dose of radioactive cobalt given with a stomach tube.

It is seen that the cobalt content per gm fresh carcass varies with diet, age and sex of the animal. The cobalt content on the fresh basis for 31- to 33-, 60- and 90-day old rats on low

cobalt diet II was found to be 0.002 p.p.m., 0.007 p.p.m. and 0.005 p.p.m., respectively. On high cobalt diet IV, the 90-day-old rat contained 0.014 p.p.m. In table 4, considering all cases, the female rat had a significantly higher cobalt content per gm fresh carcass than her brother. Underwood and Elvehjem ('38) were unable to demonstrate any difference in cobalt content of rats fed mineralized cobalt-low milk and a stock diet. Cobalt storage on excessive cobalt dosing had already been shown (Josland and McNaught, '38).

It had been known that the cobalt requirement of the rat, if any, must be very small. Underwood and Elvehjem ('38) and Underwood ('40) came to the conclusion that the cobalt requirement of the rat was less than 0.6 μg and 0.4 μg per rat per day, respectively. From the data obtained from the present research, the cobalt requirement of the albino rat has been calculated to be less than 0.03 μg per rat per day. This would amount to less than 0.7 μg of cobalt per 1000 Cal.

SUMMARY

Five diets of varying ratios of iron, copper and cobalt were fed to young healthy albino rats and the effect of the three metals on food intake, growth, hemoglobin level and metal storage and retention determined. The results were as follows:

1. Low levels of iron or copper decreased food intake, growth and hemoglobin level, but 0.003 p.p.m. to 0.08 p.p.m. of cobalt in the diet showed no difference in these responses.
2. Tentatively, cobalt retention, at levels characteristic of the food supply, was calculated. Cobalt retention on low cobalt diets (0.003 p.p.m. cobalt) was 30 to 42%, while retention on high cobalt diets (0.08 p.p.m. cobalt) was 3.3 to 4.9%.
3. The cobalt content per gm of fresh rat carcass varied with the diet, age and sex of the animals.
4. Iron and copper did not affect cobalt retention nor cobalt markedly iron and copper retention.
5. The iron and copper in the basal milk diet were relatively poorly utilized by the rat. Added copper doubled this

iron retention, while added iron had only a small or insignificant effect on this copper retention, within the range of iron used in these experiments.

6. The body conserved its iron with relatively greater efficiency than its copper.

7. While anemic animals retain more metal than normal animals on the same diet, a normal animal becoming anemic on a copper-low diet retains less copper than a litter mate in better condition on a good copper diet.

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FACTORS AFFECTING THE OCCURRENCE OF HEMORRHAGIC KIDNEYS DUE TO CHOLINE DEFICIENCY

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The hepato-renal syndrome of choline deficiency is now a well recognized entity in the albino rat. While fatty infiltration of the liver with its sequelae of necrosis and cirrhosis may be produced in rats of any age, renal lesions have only been observed consistently when weanling rats were offered diets which were choline free and of low methionine content but adequate in all other respects. Cholesterol, cystine and a high fat intake increase both the incidence and severity of the disease. This situation has been amply reviewed by Griffith ('41). There are also a few references to such lesions in older animals but these have not been consistent. From the work of Griffith it has seemed that such lesions are most likely to occur at that time when the rate of growth of the animal is at a maximum and when the phospholipid turnover in the kidney is greatest. These concepts have been substantiated by the work of Engel and Salmon ('41) and of Patterson, Keevil and McHenry ('44). The present paper records some observations concerning the effects of other dietary factors and lipotropic substances on the production of such lesions in young rats and the occurrence of these lesions in older rats.

EXPERIMENTAL

All the rats used in these studies were males of the Vanderbilt strain (Wolfe et al., '38). After weaning they were grown to a weight of 50 gm on a commercial stock chow and then transferred to the experimental diets. They were housed either in separate cages or in "group" cages, 4 rats per cage. Unless otherwise stated, all rats which did not die earlier were killed by decapitation on the eighth experimental day. Liver fat determinations were performed in the manner previously reported (Handler and Dann, '42). A few kidneys from each group were bisected and fixed in formalin, then sectioned and stained with hematoxylin and eosin. Complete histopathological descriptions will not be presented since the lesions, when found, so closely resembled those described by Christensen ('42).

Food consumption was measured daily. However, the figures in the table were calculated only for the first 5 days on the experimental diet since many animals, on becoming ill on the sixth day or later completely stopped eating and comparison of food intake for the entire experimental period with animals which did not show kidney lesions would not be valid. The food intake for rats in group cages was, of necessity, calculated as one-quarter of the food disappearing in each group cage. All values in the tables are expressed in terms of one rat for the 5-day period.

The various diets used are summarized in table 1. The additions noted in table 2 were actually substituted for an equivalent amount of the dietary carbohydrate. In addition all diets except K_{10} contained, in mg per kilo, thiamine hydrochloride 5, riboflavin 10, calcium pantothenate 40 and pyridoxine 3.

The system of grading kidney lesions requires some explanation. One plus (+) indicates lesions in only one kidney, usually only a slight mottling and almost invariably this was in the right kidney. Two plus (++) indicates some gross lesions in both kidneys. Three plus (+++) indicates the full blown disease with two, large, hyperemic, almost spleen

colored kidneys while four plus (++++) has been used to indicate the existence of hemorrhage extending beyond the kidney and into the dorsal wall of the peritoneum. This system is crude but was quite helpful in comparing the effects of different dietary regimes.

TABLE 1

Composition of diets.[1]

	K_1	K_2	K_3	K_4	K_5	K_6	K_7	K_8	K_9	K_{10}	K_{11}	K_{12}	K_{13}
Casein	15	15	15	15	15	10	8	6	4	10	12	10	12
Sucrose	58	—	—	—	—	—	—	—	—	84	71	67	57
Starch	—	—	58	—	—	63	65	67	69	—	—	—	—
Glucose	—	58	—	—	—	—	—	—	—	—	—	—	—
Lactose	—	—	—	58	—	—	—	—	—	—	—	—	—
Galactose	—	—	—	—	58	—	—	—	—	—	—	—	—
Cotton oil	15	15	15	15	15	15	15	15	15	—	—	15	15
Salts [2]	5	5	5	5	5	5	5	5	5	4	4	4	4
Crisco	—	—	—	—	—	—	—	—	—	—	10	—	5
Cod liver oil	5	5	5	5	5	5	4	4	4	—	—	4	5
Cholesterol	1.0	1.0	1.0	1.0	0.5	0.5	0.5	0.5	0.5	—	0.5	—	1.0
Cystine	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	—	0.4	—	0.5
Cellulose	—	—	—	—	—	—	—	—	—	2.0	2.0	—	—
Sulfasuxidine	—	—	—	—	—	—	—	—	—	1.0	1.0	—	—

[1] The additions noted in table 2 were substituted for an equivalent amount of dietary carbohydrate. In addition all diets except K_{10} contained, in mg/kg, thiamine 5, riboflavin 10, calcium pantothenate 40 and pyridoxine 3.

[2] Hubbell, R. G., L. B. Mendel and A. J. Wakeman, 1937, J. Nutrition, vol. 14, p. 273.

Production of hemorrhagic kidneys in young rats

When rats were housed in single cages the diet K_1, which was patterned after those described by Griffith ('41), proved most suitable for the production of kidney lesions. As shown in table 2, all rats on this basal diet showed some sign of kidney damage. However, when the animals were housed in group cages during the experimental period both the incidence and severity of the disease was markedly less than in the single cages, although liver fat concentrations and food intake were of the same magnitude in both groups. The only

explanation which has suggested itself is that the practice of coprophagy provided the rats in group cages with sufficient choline (or source of labile methyl groups) to protect the kidney against injury but not enough to reduce liver fat concentrations effectively. Evidence suggesting that some synthesis, presumably intra-intestinal, of such compounds does occur has recently been furnished by du Vigneaud et al., ('45).

In an attempt to increase the demand for methionine and therefore choline, nicotinamide was added to the diet at a concentration of 0.5%. The rationale for this procedure has

TABLE 2

Effects of various dietary factors on incidence of hemorrhagic kidneys when used as supplements to the basal ration K_1.

SPECIAL SUPPLEMENT	NO. RATS	FOOD INTAKE	LIVER FAT	HEMORRHAGIC KIDNEYS 4+	3+	2+	+	0	TYPE OF CAGE
		gms	%						
.....	24	40	14.7	1	15	7	1	0	Single
.....	24	36	16.0	0	0	6	8	10	Group
0.5% Nicotinamide	12	21	18.4	0	4	4	3	1	Group
0.5% Nicotinamide	12	22	20.2	0	2	4	3	3	Single
0.25% Inositol	24	37	12.8	17	6	0	1	0	Single
0.25% Inositol	12	34	11.6	1	7	2	1	1	Group
0.25% Inositol + 0.5% nicotinamide	24	22	13.1	8	7	5	3	1	Group
α-Tocopherol 500 μg/day	12	36	15.9	0	7	4	0	1	Single
α-Tocopherol 500 μg/day + 0.25% Inositol	12	38	9.7	0	2	3	3	4	Single
1% Lipocaic	24	35	9.1	0	1	7	5	9	Single
2% Meat extract	12	40	6.1	0	0	0	0	12	Single
5% Liver extract	12	39	5.4	0	0	0	0	12	Single
Biotin 20 μg/day + folic acid 20 μg/day	12	39	17.1	0	6	5	1	0	Single
0.15% Choline	24	41	8.7	0	5	4	8	8	Single
0.25% Choline	24	42	4.8	0	0	3	6	15	Single
0.5% Nicotinamide + 0.35% methionine	12	30	7.7	0	1	2	4	7	Group
0.25% Choline + 0.5% nicotinamide	24	26	11.4	0	7	2	8	8	Group
0.5% Choline + 0.5% nicotinamide	24	29	6.1	0	0	0	2	22	Group
Choline 6 mg/day + 0.25 inositol	12	37	8.7	2	3	4	1	2	Single

been discussed by Handler and Dann ('42). This resulted in a considerably increased incidence of hemorrhagic kidneys in the rats in group cages but actually diminished the number seen in single cages. The latter effect appears to have been due to the diminution of food intake caused by the nicotinamide induced methionine deficiency.

Effects of various lipotropic and alipotropic factors

The effects of various materials which have been suggested as having lipotropic or alipotropic activity were then tested. The results are also included in table 2. While somewhat reducing the liver fat concentration, inositol actually appeared to increase the incidence and severity of the renal syndrome. In many inositol fed animals the gross hemorrhage which is primarily intra and subcapsular appeared to spread out so that the entire dorsal wall of the peritoneum appeared hemorrhagic. Such animals also invariably showed hemorrhage in the pericardium, lungs and eyesockets as well. These extensive lesions were seldom seen on diets which did not contain inositol.

Tocopherol deficient chicks exhibit capillary damage which is manifested as a generalized exudative diathesis which is, however, prevented by the administration of inositol (Dam and Glavind, '42). Of itself, tocopherol exerted no activity in the present experiments. Tocopherol plus inositol, however, significantly decreased the incidence of hemorrhagic kidneys below that seen in the control groups, in striking contrast to the action of inositol alone. This combination also exerted significant lipotropic action in the liver. Since inositol has been implicated as an active component of "lipocaic" preparations (Abels et al., '43; Gavin and McHenry, '41 a), it was thought of interest to test its activity against choline deficiency hemorrhagic kidneys. Lipocaic was prepared by the method of Clark et al. ('39) from frozen beef pancreas obtained commercially and was included as 1% of the diet. At this level the preparation proved quite efficacious in preventing hemorrhagic kidneys. Unfortunately, the choline con-

tent of the preparation, determined by colorimetric estimation of the reineckate, permitted a daily intake of about 1.8 mg of choline. This level of choline plus 0.25% inositol had relatively little effect on the incidence of renal lesions. However, it cannot be stated whether the activity of the lipocaic was a direct one on the kidney, a sparing action in the liver or, perhaps, due to more complete liberation and availability of the methionine in the dietary casein (Chaikoff et al., '45). We are now attempting to obtain a choline free preparation of lipocaic to test it in this manner. It should be noted that the lipocaic also effectively prevented the accumulation of liver fat as well.

The fatty livers of depancreatized dogs maintained on a diet of lean beef and sucrose do not occur when the insoluble residue from the preparation of commercial meat juice extract are fed, but are manifested when the meat extract is included in the diet (Ralli and Rubin, '42). It seemed of interest to test the effect of meat juice by the present technique and it was found that when meat extract [1] was included as 2.5% of the diet only normal livers and kidneys were found. However choline analysis revealed that the preparation was providing about 17 mg of choline per day which, even in the presence of the inositol may well have been sufficient to account for the results.

Because of the suggestion that biotin increases the demand for lipotropic factors (Gavin and McHenry, '41 b) the effects of biotin,[2] crystalline folic acid [3] and a liver extract prepared according to the directions of McHenry and Gavin ('40) were tested. Neither biotin nor folic acid nor a combination of the two appeared to have any effect. The liver extract, rather than increasing the demand for lipotropic factors, actually prevented the appearance of hemorrhagic kidneys. This again was undoubtedly due to its choline content as it provided at least 11 mg of choline and an unknown amount of methionine

[1] Valentine's.
[2] Merck.
[3] Lederle.

per day. The actual roles of biotin, folic acid and inositol in the homeostatic control of liver fat concentrations has been discussed elsewhere (Handler, '46).

Influence of the dietary carbohydrate on the incidence of hemorrhagic kidneys

Griffith ('41) has stated that starch used in place of sucrose in synthetic diets prevented the appearance of hemorrhagic kidneys although no data were presented illustrating the point. We have tested the effects of substituting starch, glucose, lactose and galactose for the sucrose of diet K_1. In contrast to Griffith's statement both glucose and starch were found to be effective in such diets. When lactose or galactose were

TABLE 3

Effects of various carbohydrates in choline deficiency. All rats were housed in single cages.

DIET	CARBOHYDRATE	NO. RATS	FOOD INTAKE	LIVER FAT	HEMORRHAGIC KIDNEYS				
					4+	3+	2+	+	0
			gm	%					
K_1	Sucrose	24	40	14.7	1	15	7	1	0
K_2	Glucose	12	38	13.9	0	7	2	3	0
K_3	Starch	12	37	15.5	0	6	3	2	1
K_4	Lactose	12	26	7.4	0	0	0	1	11
K_5	Galactose	12	24	8.1	0	0	0	0	12

used no kidney lesions were found and further, there was only a relatively small increase in liver fat concentration. The daily food consumption was lower on these two diets, and it seems possible that this may account for these effects. While it is not shown in the data (table 3), those rats which ate most and did gain weight showed the largest liver fat concentrations. No rats on these diets ate or grew as well as those on the other diets. The general nutritional inadequacy of lactose as the sole carbohydrate in the diet of the rat has already been reported by Ershoff and Deuel ('44) who found that young rats die after 2 to 3 weeks on such diets. An attempt to determine the underlying mechanism of this nutritional failure will be described in a subsequent report.

Delayed appearance of hemorrhagic kidneys

While attempting to determine the optimal dietary conditions for the production of dietary hepatic cirrhosis, studies were made in which various levels of casein were tried (Handler and Dubin, '46). The diets used are summarized in table 1. At casein levels of 15, 10 and 8% (diets $K_{1, 6, 7}$) the incidence of deaths due to kidney damage between the sixth and thirteenth day were 65, 40 and 25% of the total number of animals, respectively. No animals died in this manner after the thirteenth day although they were maintained for 120 days on the same diets. When casein was fed as 6% of the diet, (K_8) no deaths occurred during the apparently critical period found in the series above. Surprisingly however, 17 out of 24 rats then died between the thirty-fourth and forty-fifth day of the experiment with acutely hemorrhagic kidneys. The survivors were sacrificed after 120 days at which time all but one were found to have the characteristic gross "frosted" kidneys indicative of kidney lesions which had healed and this was confirmed by examination of the sections. A second group of 24 animals were placed upon the same diet and groups of 6 were sacrificed at weekly intervals. No kidney lesions were noted in any but the last group sacrificed (28 days). It should also be added that no lesions were found when 4% casein was fed (K_9).

A delayed development of renal damage due to choline deficiency has also been noted under other conditions. These have been described elsewhere (Handler, '46) and so merit only brief mention in the present report insofar as is pertinent to the discussion. Male rats were fed diet K_{10} which contained 10% casein and no fat or B vitamin supplement for 21 days after they had been grown to a weight of 80 gm on a stock ration. They were then transferred to diet K_{11} which contained 12% casein, 10% crisco and adequate B vitamins including biotin and folic acid and sacrificed after 8 days. Nine out of 12 animals showed varying degrees of renal damage and all showed markedly fatty livers. Choline alone

was sufficient to prevent the renal lesions but did not quite produce normal liver fat concentrations. Inositol plus tocopherol also reduced the incidence of the kidney lesions and also considerably diminished the liver fat concentrations. While this appearance of hemorrhagic kidneys is not delayed in the same sense as those in the preceding experiment, it does again demonstrate that there is no critical age for the development of such lesions, but rather only a critical situation which may be produced or occur at any age.

Hemorrhagic kidneys in unilaterally nephrectomized rats

The renal lesions of choline deficiency have only occasionally been noted to develop in mature rats. To check this, diets K_1 (15% casein) and K_7 (8% casein) were each offered to a group of 12 rats after they had attained a weight of 250 gm on a stock ration. The animals were sacrificed after 100 days. Four of the rats on K_7, exhibited grossly cirrhotic livers and all livers in both groups were extremely fatty although quantitative estimations were not performed. All the animals on K_1 were found to have normal kidneys and 3 rats on K_7 appeared to show slight renal damage.

Forty adult male rats, all of which weighed more than 250 gm were then fed diet K_{12} (10% casein) for 6 weeks to deplete them of any choline or labile methyl reserves. They were then subjected to unilateral nephrectomy under ether anesthesia. In each case, the right kidney was removed as this is normally slightly larger than the left kidney (see table 4). They were then changed to diet K_{13} (12% casein, 20% fat) and groups of 10 were sacrificed after 8, 10, 12 and 14 days. The results and comparison with control animals on a stock regime are shown in table 4. It will be seen that hemorrhages were noted in all groups but the critical period appeared to lie between the tenth and fourteenth day after nephrectomy. Actually 4 rats were found dead on the thirteenth day with severe renal lesions and are included in the table with the group sacrificed on the fourteenth day. The

large size of the kidneys noted in these rats was undoubtedly due to a combination of hypertrophy and hyperemia such as that seen in choline deficient weanling rats on such diets.

As a control, 12 rats of the same size maintained on a stock ration were subjected to unilateral nephrectomy in the same fashion. Two weeks later they were placed on diet K_{13}. All rats survived for another 6 weeks when they were sacrificed. Moderate lesions were found in only one rat.

TABLE 4

Hemorrhagic kidneys in unilaterally nephrectomized rats. Each value is a mean for a group of 10 rats.

STATE OF RATS	DIET	TIME	LEFT KIDNEY WEIGHT	RIGHT KIDNEY WEIGHT	HEMORRHAGIC KIDNEYS			
					3+	2+	+	0
		days	*gm*	*gm*				
Normal	Stock	0	0.61	0.67	0	0	0	10
Right kidney removed	Stock	8	1.04	0.71	0	0	0	10
Right kidney removed	Stock	14	1.17	0.70	0	0	0	10
Right kidney removed	K_{13}	8	1.08	0.66	0	0	3	7
Right kidney removed	K_{13}	10	1.19	0.72	0	1	4	5
Right kidney removed	K_{13}	12	1.38	0.70	0	3	3	4
Right kidney removed	K_{13}	14	1.70	0.74	3	3	2	2

DISCUSSION

The data presented show that renal lesions due to choline deficiency may occur or be induced at any age and are most likely to occur when the food consumption, growth rate and lipid turnover are maximal. Ordinarily, the lipid turnover in the kidney probably parallels that in the entire animal. During the period of hypertrophy after unilateral nephrectomy in adult animals, however, it appeared that the demand for lipotropic factors was at least as great as in the young growing rat and when these were not provided in the diet the typical picture of choline deficiency renal damage was found in the remaining kidney. Further, renal damage due to choline deficiency appears to be an acute rather than a chronic cumulative process. The study of delayed appearance of such lesions on the 6% casein diet (K_8) confirmed this concept with regard to the morphological aspect of the kidneys, yet demonstrated

that such damage may occur at any time during the life cycle if proper conditions exist, although it is difficult to state precisely what these conditions are.

Despite its lipotropic action in the liver, inositol did not afford any protection against renal damage, but, if anything, appeared to increase both the severity and incidence of the disease. On the other hand a combination of inositol and tocopherol offered some protection. A lipocaic preparation also appeared to offer a greater degree of protection than was warranted by its choline content. It cannot be said whether this was due to a combination of factors or to an actual chemical entity which has been designated as lipocaic. If the preparation contained an appreciable amount of tocopherol and inositol this might have been responsible for the observed activity. This would parallel the inositol-tocopherol relationship in preventing the capillary damage and exudative diathesis of vitamin E deficiency (Dam and Glavind, '42). Dam ('44) has stated that no tocopherol was found in an alkaline hydrolysate of a lipocaic preparation but it is doubtful that any tocopherol could have remained after such drastic treatment nor would it necessarily have split an inositol-tocopherol ether such as that proposed by Milhorat and Bartels ('45).

SUMMARY

Rats housed in group cages failed to develop the renal lesions of choline deficiency on a diet which was quite effective when the rats were in single cages. The addition of nicotinamide to this diet resulted in renal lesions even in the rats housed in group cages. While inositol exerted lipotropic activity in the liver, it appeared to increase slightly the incidence of hemorrhagic kidneys due to choline deficiency. A combination of inositol and tocopherol as well as a lipocaic preparation significantly decreased the incidence of such lesions while biotin and folic acid were without effect. Weanling rats on a diet containing 6% casein did not develop hemorrhagic kidneys until after 35 to 45 days, in contrast to rats receiving diets of higher protein concentration which

develop such lesions in 6 to 10 days. While few adult rats developed renal lesions on choline deficient rations, choline deficient adult rats subjected to unilateral nephrectomy uniformly showed such lesions after 10 to 14 days, the period during which the remaining kidney hypertrophied. When adult nephrectomized rats were placed on a choline deficient regime 2 weeks after the operation, renal damage was observed in only one of 12 animals although they were continued on the diet for 6 weeks.

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A COMPARISON OF FOUR METHODS FOR STUDYING THE URINARY EXCRETION OF THIAMINE

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Excretion of thiamine has been used in various ways as an indication of the state of nutrition with respect to thiamine. The purpose of the work reported here was to compare the results of four methods for studying urinary excretion of thiamine in subjects on a liberal intake of thiamine. The tests used were: (1) basal 24-hour excretion, (2) percentage of intake excreted, (3) response to a 5-mg oral test dose, and (4) response to a 1-mg intramuscular test dose. The level of intake was that recommended for adults by the Food and Nutrition Board of the National Research Council at the time the study was conducted (National Research Council, '43). Several studies have been reported in which investigators have used one or two of these tests and have correlated their figures with the appearance or disappearance of physical symptoms of thiamine deficiency. Melnick ('44) and Holt ('44) have reviewed most of these investigations. No study has been reported in which these four urinary excretion tests were conducted during the same experiment on normal subjects whose intake of thiamine was controlled and constant.

[1] The data in this paper are taken from the thesis presented by Helen H. Gifft to the faculty of the Graduate School of Cornell University in fulfillment of the requirement for the M.S. degree, June, 1944. Further details will be found in the thesis.

EXPERIMENTAL PROCEDURE

Four subjects were maintained on a controlled diet for a period of 44 days, January 6 through February 18, during which time their urinary excretion of thiamine was measured. The level of thiamine intake from food, approximately 600 μg per 1000 cal. as calculated from published analyses,[2] remained constant throughout the study. On the first and last days oral test doses of 5 mg of thiamine were administered with breakfast. On the seventh, thirteenth, and forty-third days intramuscular test doses of 1 mg of thiamine were administered just before breakfast.

TABLE 1

The sex, age, height, weight, calorie and thiamine intake of the four subjects.

SUBJECTS	SEX	AGE	HEIGHT	WEIGHT	CALORIES [1]	THIAMINE INTAKE FROM FOOD [1]	AVERAGE TOTAL THIAMINE INTAKE/ DAY FOR 43 DAYS [2]
			inches	*lbs.*		*mg*	*mg*
A.M.	F	23	67½	136	2500	1.5	1.7
D.L.	F	30	66	143	2500	1.5	1.7
H.G.	M	26	71	133	3000	1.8	2.0
E.K.	M	24	69	154	3500	2.1	2.3

[1] Approximate calorie and thiamine content of the diets estimated from published analyses. See footnote [2].

[2] 5-mg oral test dose given on final day of experiment was not included since it did not affect nutritional status during the experiment.

Data concerning the subjects are given in table 1. Physicians of the Department of Clinical and Preventive Medicine at Cornell University found all subjects in good health, that is, that no condition was present which, so far as is known, influences the metabolism of thiamine.

To insure good stores of thiamine at the beginning of the experiment, subjects were given vitamin B complex capsules

[2] Food tables used were: Chatfield, C. and G. Adams, U. S. Dept. Agric. Circ. 549, 1940; Hewston, E. M., and R. L. Marsh, U. S. Dept. Agric. Misc. Pub. 505, 1942; Munsell, H. E., Milbank Mem. Fund Quart., vol. 21, p. 102, 1943. Values for riboflavin content of the wheat germ were obtained from the manufacturer. Analyses made by Doris N. Vidmar 4 months after the conclusion of the experiment indicated that thiamine values were approximately 500 μg per 1000 cal. at that time.

for 6 days preceding the experimental period. Each daily supplement furnished 4.5 mg of thiamine hydrochloride, 6 mg of riboflavin, 0.75 mg of pyridoxine hydrochloride, 30 mg of nicotinamide, and 9 mg of calcium pantothenate. No supplement was given on the day just preceding the experimental period.

Urine was preserved with 15 ml of glacial acetic acid per 24-hour specimen. Determinations were made semi-weekly on 3-day pooled samples by the thiochrome method of Hennessy and Cerecedo ('39) as modified by Hennessy ('41). Recovery values were in the range of 93 to 101%.

The experimental diet was planned so that it would be adaptable to different caloric requirements and would furnish adequate amounts of all nutrients except ascorbic acid.[3] The basal diet furnished approximately 1000 cal., 600 μg of thiamine, and 1400 μg of riboflavin. It consisted of 200 gm of evaporated milk, 100 gm of canned carrots, 100 gm of ground beef chuck, 100 gm of canned pears, 75 gm of dried prunes, 100 gm of canned green beans, 20 gm of Instant Ralston cereal, 20 gm of roasted peanuts, and one medium egg. No foods were allowed ad libitum but additions to the basal diet were planned in units. Each unit addition contained biscuits, cookies, butter, sugar, and wheat germ in amounts to furnish approximately 500 cal., 300 μg of thiamine, and 90 μg of riboflavin. Each subject was allowed to decide how many of these unit additions he needed to satisfy appetite and energy requirements, and throughout the experiment kept his food intake constant as nearly as could be determined by calculation. The calorie and thiamine intakes of each subject are shown in table 1. The diets as eaten furnished slightly more than 1000 μg of thiamine per 1000 non-fat cal. Approximately 40% of the calories were supplied by fat.

[3] Vitamin C metabolism studies which were being conducted concurrently on the same subjects necessitated a diet low in ascorbic acid, but daily supplements of synthetic ascorbic acid made the level of intake 53 mg. The subjects were saturated with ascorbic acid during the pre-experimental period.

RESULTS AND DISCUSSION

Basal 24-hour excretion

The results of the determination of urinary thiamine excretion for the four subjects of this study are shown in table 2. Note that basal urinary excretion values tended to decrease for all subjects as the study progressed. This downward trend was sharp at first and was not even temporarily interrupted by the administration of 1-mg intramuscular test doses on the seventh and thirteenth days of the period. In each case the value for the analysis just preceding the test dose was higher than for the 2- or 3-day period following the test dose day. Values at the beginning of the third week of the study were from 31 to 52% of those for the second day of the experiment. During the last 2 weeks the values had practically reached a plateau, as the maximum difference between the high and low values for any subject did not exceed 16%. The comparatively rapid decrease in urinary excretion evinced in the first 2 weeks can probably be explained by the fact that the subjects took vitamin B complex capsules for 6 days in the pre-experimental period. Melnick et al. ('39) noted that their subjects excreted unusually large amounts of thiamine after periods of excessive intake. Mason and Williams ('42) also observed elevated values for several days after ingestion of large amounts of thiamine. This experience suggests that when saturation with thiamine is to precede an experiment, an interval of 2 weeks might be allowed on a diet adequate but not excessive in thiamine, after saturation and before beginning the experiment proper.

Because of the marked effect of preliminary saturation with thiamine on the urinary excretion for the first 2 weeks of the study, average basal 24-hour excretion values and average percentage of intake excreted were calculated for the last 4 weeks of the study (table 3). These average basal 24-hour excretion values were: 197 μg for A.M., 135 μg for D.L., 195 μg for H.G., and 210 μg for E.K. In general, basal excretion values for three of the subjects, A.M., H.G., and E.K., tended

TABLE 2

Urinary excretion of thiamine of subjects on an intake of approximately 600 μg per 1000 cal. (μg per 24 hours).

DAY OF EXPT.	TEST DOSES	A.M.		D.L.		H.G.		E.K.	
		Basal excretion	Response to test dose	Basal excretion	Response to test dose	Basal excretion	Response to test dose	Basal excretion	Response to test dose
1	5-mg oral		1456		1652		1855		1971
2		616		562		534		564	
5		384		301		362		488	
6		406		269		360		429	
7	1-mg intramuscular		518		540		544		657
8–9		390		213		319		327	
10–12		327		216		271		365	
13	1-mg intramuscular		494		290		468		528
14–16		292		173		225		292	
17–19		254		184		270		282	
21–23		227		155		233		254	
24–26		178		148		208		224	
28–30		200		139		198		222	
31–33		192		118		182		222	
35–37		162		116		155		194	
38–40		173		100		163		188	
41–42		190		118		153		197	
43	1-mg intramuscular		328		193		281		403
44	5-mg oral		792		819		752		1111

to be similar, while D.L. consistently excreted less than the others. The lowest value for D.L., 100 μg per 24 hours on a 3-day pooled sample, approached the "critical level", 90 μg per 24 hours, which is the lowest figure considered by Mason and Williams ('42) to indicate adequate nutrition. All other values were well above this "critical value".

TABLE 3

Summary of results.

URINARY EXCRETION TEST	SUBJECTS			
	A.M.	D.L.	H.G.	E.K.
Basal 24-hr. excretion [1] (*μg/day*)				
Range	254–162	184–100	270–153	282–188
Average	197	135	195	210
% of intake excreted [1]				
Range	17–11	12–7	13–9	15–9
Average	13	9	11	10
Response to 5-mg oral test dose (*% recovery*) [2]				
1st day	29	33	37	40
44th day	16	16	15	22
Response to 1-mg intramuscular test dose *% recovery* [3]				
6th day	12	30	20	28
13th day	19	10	22	20
43rd day	14	8	13	21
μg/day				
6th day	518	540	544	657
13th day	494	290	468	528
43rd day	328	193	281	403

[1] For final 4 weeks of experiment.

[2] Percentage of test dose excreted in 24 hours following administration.

[3] Extra excretion obtained by subtracting basal excretion from the value for the 24-hour collection period, and then expressed as percentage recovery of the test dose. Basal excretion was interpolated from the values for the preceding and succeeding analyses.

Percentage of intake excreted

Percentage of the intake excreted by the four subjects of this study is shown in table 3. The range of values for male subjects was 9 to 15%. Melnick et al. ('39) noted values of 15 to 27% for normal men, and Jolliffe et al. ('39) observed that five men excreted 13.1 to 25.7% of their intake on a diet estimated to contain 564 μg of thiamine per 1000 cal. The range for the two female subjects of this study was 7 to 17% as compared with values of 9 to 31% observed by Melnick et al. for normal women.

Response to 5-mg oral test dose

Responses to the 5-mg oral test doses administered on the first and last day of the study are shown in table 3. Recovery was calculated by the method most commonly used, that is, the total thiamine excreted in 24 hours following administration of the dose was used to calculate the percentage recovery of the dose. The larger excretions on the first day were undoubtedly caused by vitamin supplements given the subjects during the week preceding the experimental period. Values for the last day ranged from 15 to 22% of the test dose. Melnick et al. ('39) found that 15 normal men averaged 14% and 10 normal women 12% excretion of a similar test dose. In another study Melnick and Field ('42) observed that 23 normal adults with good stores excreted 7 to 30% and 14 normal adults with poor stores excreted 1 to 7%. Six other normal adults studied by Melnick ('42) excreted 13 to 20.8% of the dose. Values for excretion of a 5-mg oral test dose by subjects at the end of this study, when compared with the aforementioned figures could be interpreted to mean that thiamine reserves were adequate.

Response to 1-mg intramuscular test dose

One-mg intramuscular test doses were administered on the seventh, thirteenth and forty-third days of the period. Responses to these doses are shown in table 3, both in milli-

grams of thiamine excreted in 24 hours, and as extra excretion due to the dose. Extra excretion, obtained by subtracting basal excretion from the value for the 24-hour collection period, was expressed as percentage recovery of the test dose. Basal excretion was obtained by interpolation.

Percentage recovery of a 1-mg intramuscular test dose by the subjects of this study is shown in table 3. These estimates are probably less accurate for the first two than for the last of the test doses, since the basal excretion fell rapidly during the first 2 weeks. Under these conditions, the figure for basal excretion on the day of the test dose, which must be interpolated to calculate percentage recovery, can be only a fair guess of what the excretion actually was. Therefore an average of two values would probably give a more suitable figure for comparative purposes than either value alone. These averages for the four subjects are: 15% for A.M., 20% for D.L., 21% for H.G., and 24% for E.K. In all cases these figures are higher than recoveries observed when a third intramuscular test dose was administered at the end of the experiment. This lower recovery is particularly notable in the cases of D.L. and H.G. The recovery value of 8% for D.L. is in the range of values observed for persons with such depleted stores that symptoms of thiamine deficiency were present (Mason and Williams, '42). The response of H.G., 13%, might indicate that some depletion had occurred, particularly since he responded to the first two test doses with much higher recoveries. Subject A.M. responded in substantially the same degree to all three of the tests. Her recovery value of 14% of the last dose might be considered a borderline figure, but probably indicated sufficient stores when interpreted in the light of her comparatively low responses to the first two doses when her reserves must have been adequate. The response of E.K. to the last test dose, 21%, was within the range of 15 to 45% recovery observed by Mason and Williams ('42) for subjects on unrestricted diets with large thiamine supplements.

Average responses to the first two test doses ranged from 415 to 592 μg of thiamine in 24 hours. These figures are comparable to those observed by Williams et al. ('42) at a similar level of intake. Excretion values for the third test dose administered 30 days after the second and at the end of the study are more comparable to those observed by Williams et al. on an intake of 400 μg per 1000 cal. The low response of D.L., 193 μg, is even less than the reported values on an intake of 300 μg per 1000 cal.

Comparison of results with four procedures

Four tests were used in this study to determine the state of the thiamine reserves, and the results of the tests were interpreted in the light of the rather meager data in the literature. On this basis, the four tests were not in agreement concerning the status of the subjects at the end of the study. Basal 24-hour excretion and responses to the 5-mg oral test dose indicated that for all subjects reserves of thiamine were adequate. This is what one would expect if the Food and Nutrition Board was justified in considering this level of intake more liberal than necessary, as is indicated by the reduction in recommended daily allowances for thiamine (National Research Council, '45). Calculation of the percentage of intake excreted suggested that subject A.M. had normal reserves but that the other three subjects had borderline or sub-normal reserves. Responses to the 1-mg intramuscular test dose suggested that only one subject, E.K., had adequate stores. No subject was judged to have normal reserves of thiamine on the basis of all four criteria.

When results for the four urinary excretion tests are considered independently of the suggested normal values in the literature, the results obtained by different methods agree reasonably well, i.e. each subject behaved fairly consistently. Throughout the study the female subject, D.L., excreted less thiamine than the other subjects and exhibited smaller responses to the test doses. The excretion values of the male subject, E.K., were in general appreciably higher than for

any of the other subjects, and his responses to the test doses given on the last 2 days of the experiment were particularly so. Possibly these subjects differed in their renal thresholds for thiamine, but our data provide no information on this point. Though all subjects maintained the same thiamine-calorie ratio in accordance with the recommended daily allowances then current (National Research Council, '43), the total intake of thiamine and calories was higher for E.K. than for any of the others. On the basis of the present recommended allowances (National Research Council, '45) which do not maintain a constant proportion of thiamine to calories, this subject had a greater margin of safety in his thiamine intake than the other subjects.

The apparent disagreement in results observed when nutritional status was judged by these four criteria suggests that the range of normal values for these tests has not yet been satisfactorily established. The range of individual variation may be so great, however, that results from four subjects are insufficient to warrant definite conclusions.

SUMMARY

Four normal adults were maintained for 44 days on a controlled diet which was estimated to furnish 600 μg of thiamine per 1000 cal. Basal 24-hour urinary excretions of thiamine were higher at the first of the period than at the end. Supplements of thiamine given during the pre-experimental period undoubtedly caused elevations in values for the first 2 weeks of the study.

Four urinary excretion tests for thiamine were studied. Values for 24-hour excretions of thiamine for the last 4 weeks ranged from 100 to 224 μg per day. Average percentages of thiamine intake excreted ranged from 9 to 13%. Responses to a 5-mg. oral test dose at the end of the study ranged from 15 to 22% recovery in 24 hours. Responses to 1-mg intramuscular test doses toward the beginning of the study ranged from 15 to 24% recovery. At the end of the study they ranged from 8 to 21% recovery, or 193 to 403 μg excretion in 24 hours.

When the urinary excretion values recorded for "normal" subjects were used as standards for comparison, the nutritional status of these subjects with respect to thiamine was not judged to be the same by all four criteria. More data are needed to establish the range of normal values for these urinary excretion tests, and their relative sensitivity.

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ERRATUM

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"The term 'Highly Variable' in table 2, page 144, refers only to the term '3–27' immediately below it."